Coupling of Trichosanthin to Dextran: Its Effects on Pharmacokinetics, Immunoactivities, and Bioactivities of Trichosanthin

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Abstract

Trichosanthin (TCS) is a purified plant protein which belongs to the family of Type I ribosome inactivating protein (RIP). It has a broad spectrum of pharmacological activities and one of these is its potent anti-HIV activity *in vitro*. Trichosanthin is a small molecular weight protein and therefore excessive renal loss and short plasma half-life is anticipated. It was shown in some clinical studies that it was also a potent antigen. Therefore, the aim of this project is to synthesis dextran-trichosanthin (DX-TCS) conjugate which combines the properties of prolonged circulatory half-life with lowered antigenicity . In this study, the dialdehyde method was used to couple TCS to dextran T40 and the pharmacokinetic, biological and immunological activities of DX-TCS compared with the native compound .

A sensitive radioimmunoassay was developed to detect plasma and urine TCS in the nanogram range. Trichosanthin was successfully coupled to dextran T40 by a dialdehyde method using TCS to dextran molar ratio of 1:25. Complete coupling was confirmed by gel filtration chromatography and the conjugate was stable upon storage for up to six months at 4°C.

In the pharmacokinetic study of the single i.v. injection of TCS (0.75 mg/kg) into normal and renal arterial ligated rats, the plasma clearance was 4.78 $\pm$ 0.57 ml/min and 0.22 $\pm$ 0.02 ml/min respectively. Injection of DX-TCS resulted in a even

lower plasma clearance of 0.06 ± 0.04 ml/min and no TCS activity was detected in urine. Despite the short half-life of TCS and the major role of kidney in elimination, recovery of TCS in urine was only 0.38 ± 0.05%. Circumstantial evidence supported that TCS was reabsorbed by renal tubular cells. Firstly, the percentage urine recovery of TCS could be increased in a dose dependent manner and could be up to 40% when 12 mg/kg TCS was injected, suggesting saturation of the reabsorption process. Secondly, simultaneously infusion of other filterable proteins such as lysozyme and haemoglobin increased TCS excretion, implying the utilization of the common endocytotic process for low molecular weight proteins. Furthermore, this reabsorption process also damaged the renal tubular cells as manifested by the depression of glomerular filtration rate after TCS injection. This renal toxicity was abolished when DX-TCS was used or by simultaneous lysozyme infusion.

The biological activities of DX-TCS were evaluated by different bioassays. Firstly, DX-TCS, like its parent compound induces mid-term abortion with decreased potency. A dose of 0.02 mg/25 g TCS induced 100% abortion while 0.4 mg/25 g DX-TCS was needed to achieve the same effect. In addition, a dose of 0.1 mg/25 g TCS was toxic and produced 33% mortality rate while 4 times as much DX-TCS did not cause any death. Secondly, the immunosuppressive ability of TCS was maintained. DX-TCS suppressed the Con A and PHA but not LPS mitogenic effect with decreased potency. Thirdly, both TCS and DX-TCS exhibited cytostatic effect on PU5 and hepatoma tumour cells with the ID_{50} at the levels of 5 μg/ml and 30

μg/ml for PU5 tumour cells and 10 μg/ml and 50 μg/ml for the hepatoma cells.

In the immunogenicity study of TCS and DX-TCS, it was found that the conjugate elicited less IgG and IgE against the molecule than that of TCS using enzyme linked immunosorbent assay (ELISA).

LIST OF ABBREVIATIONS

AUC	area under curve
BSA	bovine serum albumin
B.W.	body weight
°C	degree Centigrade
CO_2	carbon dioxide
Cl_p	plasma clearance rate
Con A	concanavalin A
Cpm	counts per minute
Da	Dalton
DNA	deoxyribonucleic acid
Dx	dextran
DX-TCS	dextran-trichosanthin
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	gram
GFR	glomerular filtration rate
Hb	haemoglobin
^{3}H-dTR	tritiated-thymidine
HIV	human immunodeficiency virus
H_2O_2	hydrogen peroxide
hr	hour
H_2SO_4	sulphuric acid
ID_{50}	50% inhibitory dose
^{125}I	^{125}iodine
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-2	interleukin-2
i.p.	intraperitoneal
i.v.	intravenous
kDa	kilodalton
kg	kilogram
l	litre
LPS	lipopolysaccharide
Lyz	lysozyme
MEM	minimal essential medium
mg	milligram
min	minute
ml	millilitre
MRT	mean residence time

$Na^{125}I$	sodium ^{125}iodide
$NaIO_4$	sodium periodate
NaOH	sodium hydroxide
ng	nanogram
NH_4Cl	ammonium chloride
NS	normal saline
OD	optical density
OPD	o-phenylenediamine dihydrochloride
PBS	phosphate-buffered saline
PEG	polyethyleneglycol
PHA	phytohaemagglutinin
pI	isoelectric pH
POPOP	1,4-bis-2-(5-phenyloxazolyl)benzene
PPO	2,5-diphenyloxazole
RIP	ribosome inactivating protein
RNA	ribonucleic acid
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second
SEM	standard error of mean
SRBC	sheep red blood cell
$t_{½}$	biological half-life
TCS	trichosanthin
UV	ultraviolet
V_{ss}	steady state distribution volume
V_z	apparent distribution volume
μCi	microcurie
μg	microgram
μl	microlitre
μM	micromolar

CONTENTS

Chapter 1: Introduction

Trichosanthin (TCS)

1. Source and Purification

Trichosanthin (TCS) (天花粉蛋白) is a plant derived protein that is purified from the root tubers of the Chinese medicinal plant *Trichosanthes kirilowii* (Curcubitaceae). It has been identified as the active ingredient of the Chinese medicine, Tian Hua Fen (1,2,3). This medicine was described as early as the 14th century in China by Li Shi-Zhen in the book named *Compendium of Materia Medica* (本草綱目). It is used traditionally in folk medicine since ancient times to induce menstruation and to expel fetal membranes (4). Nowadays, it is still being used in China to induce abortions, particularly those in the second trimester (5,6), and to treat choriocarcinoma, hydatidiform moles, and ectopic pregnancy (6,7).

The multistep purification procedure of TCS involved homogenization of root tubers, acetone fractionation, ammonium sulphate precipitation, ion-exchange chromatography on CM-sepharose and preparative agarose electrophoresis. The homogeneity was demonstrated by immunoelectrophoresis and SDS-polyacrylamide gel electrophoresis (8).

2. Physicochemical Properties

Trichosanthin is a single polypeptide chain with a molecular mass of 26 kDa; containing no carbohydrates, phosphorous and cysteine residue. It is basic protein with an isoelectric point of 9.4 (9,10). The complete amino acid sequence of TCS was first reported by Wang in 1985 (9), that it consisted of 234 amino acid residues. Later in 1989, Collins *et al* reported a modified version which was substantially different from the previous one (11). It is quite clear now that TCS is synthesized first as a prepro-protein consisting of 289 amino acids. The first 23 residues comprise a putative signal peptide. The last 19 residues comprise a carboxyl extension that has never been reported to be associated with the mature protein and hence may be processed in the endoplasmic reticulum or Golgi apparatus of cells producing TCS. The mature protein thus consists of 247 amino acids.

Study of its secondary structure by circular dichroism showed that the TCS molecule consisted of about 30% helix, 40-60% ß-sheet and no ß-turns (12). Its three-dimensional structure had been determined by X-ray crystallography (13) and the gene encoding TCS was isolated and the DNA sequence determined (14,15).

3. Biological Properties

I. Ribosome inactivating property

Ribosome inactivating proteins (RIPs) is a large and widely distributed class of plant proteins that are potent inhibitors of eukaryotic protein synthesis. They are usually present in high concentrations but their biological significance in plants is still puzzling to plant physiologists (16,17).

There are 2 types of RIPs: the single-chained Type I proteins (e.g saporin) and the double-chained Type II proteins (e.g. ricin, abrin). Type I RIPs are single polypeptide chain with molecular mass of about 30 kDa and strongly basic (pI often $\geq$ 9.5). Type II RIPs consists of a ribosome-inactivating A chain with molecular mass of about 30 kDa, pI between 4.8 and 8, linked by a single disulphide bond to a B chain which exhibits lectin-like properties. The latter subunit binds the protein to D-galactose present on the eukaryotic cell surface. Thus, the B chain facilitates the entry of the A chain into the cytosol where it exerts the ribosome inactivating action. The molecular mass of the whole molecule is typically in the order of 60 kDa.

In the past few years, there was a lot of evidences to suggest that TCS is actually a Type I ribosome inactivating protein. Trichosanthin has potent activity in

inhibiting protein synthesis in a reticulocyte lysate system (18,19,20). Zhang and Wang revealed a remarkable sequence homology of 56% between TCS and ricin A chain when both identical and conservative residues are considered (21). The structural similarities of the ricin A chain and TCS were further demonstrated by circular dichroism analysis (22). There was also tertiary structural similarity between TCS and ricin A chain (23,24). Furthermore, based on the sequence homologies between these two proteins, the primary sequence of TCS was fitted to the backbone structure for ricin A chain to generate energy-minimized molecular model (11).

As demonstrated by the A chain of ricin, the action of RIPs is on the 60 S ribosomal subunit. It catalytically hydrolyses a single N-glycosidic bond of the adenosine residue at position 4324 (A-4324) in 28 S rRNA of rat liver. After releasing the adenine from the rRNA, the formation of the complex between ribosome, elongation-factor-2 (EF-2) and GTP which is the first event during protein synthesis is inhibited. Consequently, this would lead to a rapid shut down of protein synthesis (25,26,27,28).

II. Abortifacient property

Tian Hua Fen, the root tubers of *Trichosanthes kirilowii* has been used in conjunction with seven to eight other Chinese herbs to induce abortion since ancient

times (2). Later in 1971, "Radix trichosanthis", a traditional Chinese drug extracted from the root tubers of *Trichosanthes kirilowii* was introduced in China for induction of midtrimester abortion with a recommended single doses of 5 to 12.5mg (29). Generally, these doses are effective for the intended clinical indication and well tolerated. The active component of Radix trichosanthis was subsequently identified as the plant protein TCS.

Mid-term abortifacient activity of TCS was also studied in several mammalian models including mice, rabbits and monkeys (3,30,31). Its mode of action was examined extensively. Xiong *et al* (32) showed that TCS exerted direct and specific injurious effects on human syncytiotrophoblasts. The abortifacient action of TCS was therefore postulated to be mediated through its direct specific action on placental trophoblasts. It was suggested that TCS may damage the syncytiotrophoblasts of the placental villi, releasing clumps of disintegrating cells into the circulation. This hinders placenta blood circulation, leading to tissue necrosis and eventually abortion (33,34,35).

Apart from mid-term abortifacient activity, TCS was also used to terminate early pregnancies (5,6). Similar effect was also found in mouse (36). The protein exerts its effect by blocking the hatching of embryos form the zona pellucida, decreasing the incidence of successful attachment of the blastocyst, reducing the

trophoblast outgrowth and disrupting the development of inner cell mass. Therefore, it does not only result in the retardation of the development of the embryos but also prevent their implantation, leading to the termination of early pregnancy.

III. Anti-tumour activity

Trichosanthin was shown to be active against the abnormal growth of trophoblastic cells in hydatidiform mole, malignant mole and choriocarcinoma, both *in vivo* (37) and *in vitro* (39,40,41,42,43). This action is believed to be related to its ribosome inactivating activity and the preferential cytotoxicity to certain types of cells such as the trophoblasts and trophoblast-derived cells in the above examples.

Clinically, trichosanthin has been used for the treatment of trophoblastic tumours. Clinical reports from hospitals in mainland China showed high cure rates (90-98%) in malignant moles using TCS alone or in combination with other chemotherapeutic drugs, herbal medicines and surgery (37,100). The prognosis of hydatidiform mole was better than choriocarcinoma when using TCS for treatment of the cancer patients.

In recent years, the possibility of producing immunotoxins (or antibody-conjugated toxins) has been studied extensively (44,45). This is of considerable

interest because such molecules can be selectively targeted to a particular cell type, such as cancer cells. In fact, TCS was successfully conjugated to an antihepatoma monoclonal antibody. The immunotoxin shows potent and quite specific antihepatoma activity *in vitro* (46). Even though the therapeutic potential of immunotoxins is attractive, there are still some problems which require unique solutions before the consideration for entry into clinical trials. These includes the stability and biodistribution of conjugates *in vivo*, the specific reactivity of the conjugates to the targeting tumours, the access and localization of conjugates in the tumour and the immunogenicity of the immunotoxins.

IV. Anti-HIV activities

Recently, TCS (also called GLQ 223 by the authors) was first shown by McGrath in 1989 to have potent activity against HIV-1 infected cells *in vitro* (47). Trichosanthin inhibits HIV-1 replication in both acutely infected T-lymphoblastoid cells and in chronically infected macrophages. In acutely infected T-lymphoblastoid cells, treatment with TCS produced concentration-dependent inhibition of HIV replications as determined by a reduction in HIV protein (p24 levels). Moreover, TCS treatment also resulted in a selective proportional decrease in the amount of viral RNA present in the treated cells relative to total cellular RNA. On the other hand, TCS treatment of monocyte/macrophages chronically infected with HIV *in vitro*

completely abrogated the HIV antigen expression and ultimately killed the HIV-infected cells in cultures. The mechanism of the selective inhibitory activity of TCS is still unknown. Although it is a type I ribosome inactivating protein, it has not been established whether the anti-HIV activity is directly related to this property. Furthermore, it seems that the viral RNA synthesis was more suspectable to TCS and therefore perhaps suggesting a possible selective effect of the drug on viral nucleic acid synthesis, processing or stability (47,48,49).

Due to the reported anti-HIV activity of TCS, it becomes an interesting candidate as a potential therapeutic agent against HIV infection. Clinical studies are actively underway. In fact, both phase I and phase II clinical study have been completed to access the safety, pharmacokinetics and tolerance of TCS in AIDS patients. The most serious problem was the numerous side effects seen with this compound such as headache, myalgia, fevers, fatigue and neurotoxicity. Therefore, the potential use of TCS clinically as anti-HIV drug still requires careful further evaluation (50,51).

4. Immunological Properties

I. Immunosuppressive effect

Trichosanthin can be considered as an agent that modulate the immune system

by suppressing a variety of immune reactions. For example, it inhibits the mitogen-induced lymphoproliferative response, the production of IL-2 by Con A activated splenocytes and the effector functions of macrophages such as phagocytic activity *in vitro*. It also depresses *in vivo* reaction such as delayed-type hypersensitivity to sheep rat blood cell (SRBC) (53,54).

II. Antigenicity and allergenicity

Trichosanthin is a plant protein which has potential immunological side effects. This may hinder the extensive pharmacological application of this protein. Indeed, anaphylaxis is observed occasionally in patients treated with the purified protein (93,94). Experimental results in animal model indicate that injection of TCS with albumin hydroxide as adjuvant into mice would result in the production of high titre of IgE.

Studies on its immunological characteristics has shown 16 monoclonal antibodies including 5 IgE, 8 IgG1, 1 IgG3, 1 IgA and 1 IgM. Competitive binding assay further indicates that these 16 monoclonal antibodies fall into 4 groups of antigenic determinants/epitopes (56).

5. DNase-like activity

It was recently demonstrated that TCS possess DNase-like activity. Trichosanthin was able to cleave the supercoiled double-stranded DNA to produce nicked circular and linear DNA. Whether this interesting phenomena of TCS on different forms of DNA substrates is relate to the mechanism of anti-HIV activity remains to be explored (52).

Dextran Conjugates

1. Advantages of synthesizing conjugates

There are many parameters such as half-life that can affect the usefulness of a biologically active component as a therapeutic agent. Factors affecting half-life of a drug in the host includes enzymes degradation, renal loss and poor chemical stability. Therapeutic use of a drug with short half-life is limited by the need to give frequent doses to the patient. It is inconvenient to the patients and large amount of drugs are required. This will create problem especially with drugs that are potent antigen. Repeated injections of these drugs to the patients would increase the risk of a hypersensitivity reaction.

As a consequence, in the past few decades, many attempts have been tried to increase the biological half-life and reduce the antigenicity of the drug through chemical modification. One of the approaches has involved coupling the drug to a carrier such as homologous albumin (95), polyethylene glycol (96) or dextran (57,58) etc.. Among these compounds which can be employed as a possible carrier of pharmacological substances is the polysaccharides, dextran which seems to possess some of the necessary qualifications that will be explained later. The exact mechanisms of the enhanced plasma half-life and reduced antigenicity of the protein by the dextran carrier are still unknown. However, it has been postulated that the

effect may be due to the protection against degradation or removal of the intact protein through changes in the electrostatic charge of the conjugated protein (59), or the increase in relative mass (60). Increase in molecular mass would also allow the protein to escape form glomerular filtration. Reduced immunogenicity may also occurred due to the blocking of the antigenic determinants on the molecule (61,62,63).

2. Dextran as carrier

Dextran is a high-molecular-weight polymer of D-glucose linked predominantly by α-D (1→6) linkages (64). It is synthesized by enzymes called glycosyltransferases on the cell surface of certain lactic acid bacteria. Several organisms produce dextrans but only *Leuconostoc mesenteroides* and *L. dextranicum* (*Lactobacteriaceae*) have been used commercially.

Dextrans have several characteristics that make it a suitable drug carriers. Firstly, it is soluble in water. Secondly, it can be obtained in a wide range of molecular sizes. Dextrans with average molecular weights of 1000, 5000, 40,000, 75,000, 110,000, 700,000, and even more are available. Thirdly, it can be activated by various chemical methods to react to form defined and stable compounds. Lastly, it lacks any significant toxicity or organ or cellular tropism. In fact, dextrans with

molecular weight of 40,000 and 70,000 are used clinically as plasma volume expander. Therefore, dextrans can be considered as essentially harmless except some higher molecular weight dextrans which are immunogenic in human (65).

3. Strategy for producing dextran conjugates

For the synthesis of any conjugates, an ideal conjugation method should (a) have minimum interference with the activity of the parent compound; (b) allow high incorporation, yet be sufficiently controllable to be tailored to the production of homogeneous conjugates with optimal carrier to drug ratios; (c) avoid formation of homopolymer of either the carrier or drug; (d) avoid aggregation of the conjugate; and (e) be technically straightforward and reproducible.

Numerous methods have been developed to activate dextran for coupling to protein molecule. For example, oxidation of the polysaccharide by periodate to form polyaldehyde dextran; preparation of an azide dextran; activation by cyanogen halides or organic cyanates to form imidocarbonate dextrans and preparation of bromo- or chlorohydroxypropyl dextran, through a reaction with bromo- or chloropropyl epoxide. All these methods allow us to synthesize drug-linked polysaccharides with a suitable molecular weight. After the reaction, the conjugates can be purified from the unreacted drug by various biochemical methods such as column chromatography

4. Examples of dextran-protein conjugates

In the search for a better blood substitute, the first dextran-protein conjugate (dextran-haemoglobin complex) was synthesized. Coupling of haemoglobin to dextran successfully prevent its filtration through the kidneys and thus prolong the half-life of haemoglobin in the circulation. The biological activity of transporting oxygen was slightly altered and the complex was found to be non-immunogenic in homologous species (66,67,68,69).

Another example is the coupling of microbial enzymes such as asparaginase to dextran. Microbial enzymes offer several possibilities for application in cancer chemotherapy. Continuous depletion of specific amino acids from body fluids by enzymatic action has been proved to be a therapeutic approach demonstrating a high degree of specificity for the neoplasm. An example is the treatment of acute lymphoblastic leukemias by the enzyme asparaginase (70). Unfortunately, microbial enzymes usually have a relatively short half-life of less than 10 hours. Some are even shown to be immunogenic in human (71). Therefore, modification of the microbial enzymes by chemically couple to dextran has been investigated in the past ten years. According to this principle, soluble dextran-asparaginase conjugates was synthesized. The plasma half-life was shown to be increased and the antigen reactivity was reduced (71,72).

Other examples include the conjugation of carboxypeptidase G (57,73,74), adenosine deaminase (75) and arginase (57) to soluble dextran. Similarly, their plasma half-lives were successfully prolonged. As a result, coupling of protein to dextran is a possible way to improve the therapeutic usage of a drug and this would allow a wider clinically application of the modified drug.

5. Dextran-trichosanthin conjugate

Although the plant protein trichosanthin has wide spectrum of pharmacological actions such as mid-term abortifacient activity, anti-tumor and anti-HIV activities etc., it is still limited in use due to some of its characteristics such as short half-life, immunogenicity and its toxicity. Trichosanthin is a low molecular weight protein with molecular weight of only 26 kDa. Therefore, a short plasma half-life is anticipated since it can be rapidly excreted by the kidneys. In order to overcome these limitations, trichosanthin is covalently couple to dextran T40 with a molecular weight of 40 kDa.

The aim of this study is to modify the protein, trichosanthin by linking it to dextran so as to expand the molecular size of it. This would retard its filtration through the kidney and result in a longer half-life. At the same time, antigenicity of this protein may also be reduced.

Chapter 2: Methodology

1. Preparation of Trichosanthin and its antiserum

Trichosanthin was purified from the root tubers of *Trichosanthes kirilowii* obtained from Kwansi Province, China as described in the reference (8). Briefly, the root tubers were first cut into small pieces, homogenized with 0.9% normal saline and filtered to give a crude extract which was then subjected to acetone fractionation, giving rise to the AP4 fraction. It was further purified by ammonium sulphate precipitation to remove the contaminant and the supernatant designated S40 was obtained. This resulting fraction was then applied to a CM-Sepharose CL-6B column and purified TCS could be obtained first by isocratic elution with 0.05 M phosphate buffer, pH 6.4 followed by gradient elution with 0-0.3 M PBS, pH 6.4. Homogeneity of the protein was demonstrated by SDS-polyacrylamide gel electrophoresis and immunoelectrophoresis. A solution at concentration of 1 mg/ml TCS was prepared by dissolving in phosphate buffer saline (PBS), pH 7.2. Protein concentration was determined spectrophotometrically using O.D. 280 nm.

The polyclonal antibodies against TCS were generated as described by Shaw *et al* (76). Antibodies were raised in 3-month-old (2.5 kg) albino rabbits using denatured TCS. Crystalline trichosanthin (100 μg in 0.25ml normal saline containing 0.5 % sodium dodecyl sulphate) was denatured by heat treatment at 80°C for 10 min.

It was then diluted to a final concentration of 200 μg/ml, and emulsified with equal volume of complete Freund's adjuvant. The rabbits first received intradermal injection of 1 ml emulsion at a number of sites on the back. Afterwards, each rabbit received a total of five booster injections, containing the same quantity of antigen but emulsified with incomplete Freund's adjuvant at 3-week interval. During this time, the activities of the antiserum were monitored by immunoblotting (77) using the ProtoBlot™ system (Promega, WI, U.S.A.). Two weeks after the last injection, the rabbit with the highest antibody titre was completely bled and the antiserum collected and stored at -20°C.

2. Preparation of iodinated Trichosanthin

Trichosanthin was iodinated by using the chloramine-T method. Chloramine-T method (78) and metabisulphite solution were freshly prepared. For the reaction mixture, 500 μCi $Na^{125}I$ (in 5 μl 0.5 M phosphate buffer, pH 7.5), 10 μg chloramine-T (in 10 μl PBS, pH 7.6) were added to 5 μg TCS (in 10 μl PBS, pH 7.6). The reaction mixture was agitated for 10 sec and the reaction stopped by the addition of 50 μg metabisulphite (in 100 μl PBS, pH 7.6). The unreacted iodide was separated from the labelled antigen by gel filtration chromatography on a Sephadex G-75 column (1 X 14 cm). Non-specific sites on the column were saturated by rinsing it with 1 ml 2% bovine serum albumin (BSA) followed by 30 ml PBS (pH 7.6). The

iodination mixture was eluted with PBS (pH 7.6), and 1.5 ml-fractions were collected in test tubes containing 0.2 ml 1% BSA and 0.1% sodium azide in the elution buffer. The peak tubes that contained the highest count of ^{125}I-TCS were used and stored at 4°C.

3. Radioimmunoassay procedure

Radioimmunoassay was performed by using a double-antibody polyethylene glycol (PEG) method under equilibrium conditions. The assay buffer used contained 0.01 M PBS with 1% BSA and 0.1% sodium azide, in which standards, antisera, normal rabbit serum and labelled antigen were diluted.

A typical reaction mixture contained 100 μl each of ^{125}I-TCS (about 20,000 cpm), 2% normal rabbit serum, standard (5-1000 ng/ml) or sample, antisera (final concentration 1:25,000) and assay buffer. Non-specific binding was accessed by substitutizing the aliquots of antisera and standard with same volumes of assay buffer. The final volume was 500 μl per tube and all tubes were made in duplicates. The assay tubes were then incubated overnight at room temperature.

In order to separate the free antigen from antibody-antigen complex, the latter was precipitated by adding 200 μl of goat anti-rabbit τ-globulin (1:40 in assay buffer

with 10% PEG 6000). After incubation for 15 min at 4°C, it was then centrifuged for 4 min at 9000 r.p.m. at 0°C. The supernatant was removed, washed once with ice-cold PBS. The radioactivity in the pellet was determined with a Kontron gamma counting system. Results were automatically calculated using a computer programme, based on log-logit transformation.

4. Coupling of Trichosanthin to dextran T40

The dialdehyde method described by Tam *et al* (66) was essentially followed to prepare the dextran-trichosanthin (DX-TCS) conjugate. In this method, 10 ml of dextran T40 (100 mg/ml) with an average molecular weight of 40 kDa was first oxidized by 1 ml of sodium periodate (120 mg/ml) and then left overnight in the dark at 4°C. Afterwards, freshly prepared sodium bisulphite (300 mg/ml) was added drop by drop until the mixture turned brown and then colourless again. The activated dextran was then dialysed against distilled water.

Coupling of TCS to dextran was done at different TCS/dextran molar ratios of 1:100, 1:50, 1:25, 1:10, 1:5 and 1:2.5, respectively. The pH of the reaction mixture was adjusted to 9.5 by adding 0.3 M sodium carbonate and the mixture was left overnight at 4°C. After the reaction, successful coupling was confirmed by gel exclusion chromatography on a Sephadex G-200 column (1.8 X 55 cm). Samples

were eluted with PBS, pH 7.6. The void volume of the column was determined using blue dextran (2 X 10^6 daltons). Four-millilitre fractions were collected and the TCS concentration of each fraction determined by radioimmunoassay as described previously.

5. Plasma protein binding of Trichosanthin

Plasma protein binding to TCS was evaluated by using Centricon-30 microconcentrator. Same quantity of TCS was either incubated with PBS or rat plasma at 37°C for 15 min. Then the mixtures filtered through a membrane with molecular cut off of 30,000 (Centricon-30). The concentration of TCS in the ultrafiltrate was determined by radioimmunoassay described above.

6. Pharmacokinetic study

The pharmacokinetics of a single i.v. bolus injection of TCS or DX-TCS were studied in male Sprague-Dawley rats weighing between 350 and 400 g. After an overnight fast, the rats were anesthetized by 4.5% sodium pentobarbital (60 mg/kg i.p.). The jugular vein was cannulated for drug injection. The carotid artery was cannulated for blood sampling and blood pressure measurement. The trachea was also cannulated. The urinary bladder was carefully dissected out and a catheter was placed

in it to collect urine. The penis was also ligated to prevent extravasation of urine. The temperature of the rats was maintained by a lamp shining directly on the rat. In some rats, the kidney was rendered non-functional by ligating the renal arteries. After the surgical procedures, a single dose of tritiated inulin (6 μCi) was given to the rats through the jugular vein. Rats were divided into 3 groups. Group 1 were normal rats injected with TCS (0.75 mg/kg). Group 2 were renal artery-ligated rats injected with TCS (0.75 mg/kg). Group 3 were normal rats injected with DX-TCS (0.75 mg/kg). Plasma samples (0.3 ml) were taken at 2, 5, 15, 25, 35, 45, 135 and 180 min after injection. Urine samples were collected at 45, 90, 135, 180 min after drug administration. Renal function was assessed by examining the plasma tritiated inulin excreted in urine.

In determining plasma and urine tritiated inulin, aliquot of 50 μl of samples were added to 10 ml of the scintillation cocktail which contained 2,5-diphenyloxazole (PPO), 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP) and Triton X-100 in toluene. The vials were then counted for a maximum of 10 min by using Beckman LS 7000 liquid scintillation system. Plasma and urine concentrations of TCS or DX-TCS were determined by the radioimmunoassay as described earlier.

7. Reabsorption of Trichosanthin

Two sets of experiment were performed to illustrate that TCS was reabsorbed by renal tubular cells. First, the relationship between various doses of TCS injections and its urinary excretion was examined. Rats were prepared according to the previous surgical procedures. Different doses of TCS (0.375 mg/kg, 0.75 mg/kg, 1.5 mg/kg, 3.0 mg/kg, 12mg/kg) were injected. The plasma concentration of TCS at 45, 90 and 135 min and the percentage of injected TCS recovered in urine during this time interval were determined.

In another set of experiment, the competitive nature of TCS with other protein for renal reabsorption was studied. In this experiment, either normal saline solution, 5 g/l (77.5 μM) of stroma-free haemoglobin solution (66) or 1.13 g/l (77.5 μM) lysozyme solution was infused (3.4 ml/hr) into similarly prepared rats. After 90 minutes of infusion, 0.375 mg/kg TCS was injected intravenously and the urinary TCS excretion was measured at 45 minutes interval by the radioimmunoassay described above. The glomerular filtration rate of the rats was estimated by tritiated inulin clearance.

8. Biological activities of DX-TCS conjugate

I. *in vivo* bioassay - Mid term abortifacient activity

Pregnant mice were prepared by the University Animal House. During the preparation, mature female mice were caged with fertile males. The presence of copulation plug in the following morning is designated day 1 of pregnancy. Assay was done by injecting various doses of TCS and DX-TCS intraperitoneally into the mice on day 12 of pregnancy. The mice were autopsied on day 14 of pregnancy. The total number of uterine implantation sites were recorded. The no. of live fetuses, dead fetuses whose hearts had stopped pulsating, and resorbing fetuses were recorded. Mice were considered aborted when the no. of dead fetuses exceeded 50% of the total implantation sites.

II. *in vitro* bioassays

A. Mitogen-induced lymphocyte transformation

Three to four inbred C57 BL/6N mice (6-8 weeks old) were killed with cervical dislocation. Their spleens were removed aseptically, minced with a pair of scissors and pressed against a sterile 60 mesh stainless steel screen with the plunger of a 5 ml plastic syringe. Large cell clumps which passed through the screen mesh were removed by centrifuging at 500 g for 10 sec. The cells were then pelleted and

resuspended in Tris-buffered isotonic NH_4Cl solution for 2-5 min to lyse the red blood cells. The cell suspension was then centrifuged at 500 g, 4°C for 5 min and washed two times with RPMI medium. The viability of the final cell suspension was determined by the trypan blue dye exclusion method (80). Spleen cells with the viability of over 90% was used. The cell concentration was also determined with a hemocytometer.

Spleen cells were cultured in flat-bottomed 96-well microtiter plates at $5X10^5$ cells/well in a final volume of 0.2 ml RPMI complete medium (RPMI medium supplemented with 10% foetal calf serum and containing 100 units/ml of penicillin G, 100 μg/ml of streptomycin sulfate and 3 μg/ml of fungizone). Mitogens at predetermined optimum concentrations were then added to cultures containing various concentrations of TCS, DX-TCS and dextran. Mitogens used include concanavalin A (Con A, 3 μg/ml), phytohaemagglutinin A (PHA, 10 μg/ml) and lipopolysaccharide (LPS, 30 μg/ml). The cultures were kept for 48 hr at 37°C in a humidified incubator containing 5% CO_2. After the culture period, each well was pulsed with 0.5 μCi of the labelled DNA precursor, [methyl-^{3}H]thymidine (^{3}H-dTR) for 6 hr. The radioactivity incorporated was harvested by a PHD cell harvester (Cambridge Tech.) on a glass fiber filter. The filter was suspended in scintillation cocktail and counted by using Beckman LS 7000 liquid scintillation system.

In a separate experiment, lymphotoxicity was checked by incubating the mouse lymphocytes with a high concentration (50 μg/ml) of TCS, DX-TCS or dextran for 48 hr. The % viability was assessed by using the trypan blue dye exclusion method (80). After the incubation period, 50 μl of cells was pipetted out and mixed with 10 μl 1% trypan blue dye. Five minutes later, cells which were stained blue as observed under the microscope were regarded as dead cells. The degree of cytotoxicity was then evaluated by comparing with the control in the absence of drugs.

B. Anti-tumor activity

The *in vitro* anti-tumor activity of TCS and DX-TCS was assessed on 2 different cell lines, PU5-1.8 and H35 obtained from ATCC. PU5-1.8 is a macrophage-like cell line derived form BALB/c mice while H35 is a rat hepatoma cell line. PU5 cells and H35 cells were maintained in 25 cm^2 culture flask in complete RPMI medium and complete minimal essential medium (MEM) respectively. They were subcultured every 2-3 days after the cells almost formed confluent monolayer. During the subculture process, the cells that attach to the culture flask were trypsinized (0.25% trypsin containing 0.01% EDTA) for 2-5 min at 37°C. Trypsinization was stopped by addition of complete medium to the trypsinized cell suspension. The cells were washed once with complete medium. About 10^5 viable cells were then resuspended in complete medium into the culture flask.

In the anti-tumour assay, different concentrations of TCS, DX-TCS or dextran were co-cultured with $5X10^3$ tumour cells at their logarithmic phase of growth in 0.2 ml complete medium in the wells of a flat-bottomed 96-well microtitre plate. After 48 hr culture, the growth inhibitory activity of TCS, DX-TCS or dextran on tumour cells was measured as described for the ^{3}H-TdR incorporation assay. Since both are of adherent cells, 50 μl of 0.1 M NaOH was added to each well to detach the adherent cells prior to the normal cell harvesting procedure.

The direct cytotoxic effect of TCS, DX-TCS or dextran on the cultured tumour cells were also studied. $5\text{-}10X10^4$ tumour cells were incubated with a high concentration (100 μg/ml) of TCS, DX-TCS or dextran for 48 hr, and cell viability was assessed by using the trypan blue dye exclusion method. Cells which were stained blue as observed under the microscope were regarded as dead cells. Cytotoxicity, as judged by cell killing can then be evaluated.

9. Immunological activities of DX-TCS conjugates

I. Binding activity of DX-TCS conjugate to TCS antibodies

The binding activities of TCS and DX-TCS to TCS antibodies were compared in this study. Competitive binding studies were performed as describe above (p.18)

to examine whether DX-TCS still retains its specific binding capacity to TCS antibodies. Binding study of DX-TCS with the dextran moiety digested by incubating the complex with dextranase at pH 7.6 and 36°C for 20 hr was done. The effect of dextran and/or dextranase on the TCS competitive binding curve were also studied by adding these substances to the assay.

II. Production of IgG and IgE antibody to TCS and DX-TCS in mice

C57 BL/6N inbred mice (6-8 weeks old) were used for the experiments. Mice in groups of five were immunized subcutaneously at the back with TCS (10 μg), DX-TCS (10 μg) and dextran in complete Freund's adjuvant on day 0. The booster injection was given on day 21 using the same antigens but with Freund's incomplete adjuvant. The bleeding of the primed mice was carried out 14 days after the booster injection. Blood was collected through retro-bulbar puncture under light ether anesthesia. Sera from each group of mice were stored at -20°C until use.

Another experiment with same immunization protocol was done for comparison in which adjuvant was not used. Parallel groups of the same strain of mice were immunized with the same antigen in vegatable oil instead. Moreover, hyperimmune mouse sera against TCS which has been characterized by radioimmunoassay was obtained from Dr. C. C. Wong as positive control.

Specific IgG and IgE antibodies against TCS, DX-TCS and dextran were detected by conventional enzyme-linked immunosorbant assay (ELISA). ELISA plates (Immunoplate, Maxisorp, Nunc) were coated overnight at 4°C with 100 μl of antigen (5 μg/ml TCS, DX-TCS or 100 μg/ml dextran) in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.6 with 0.1% sodium azide). On the following day the plates were washed three times with washing buffer (PBS, pH 7.2 with 0.05% v/v Tween-20) and then blocked for 1 hr at 37°C with 200 μl/well blocking solution (washing buffer with 1% BSA). After blocking the plates were again washed, then blotted dry. Positive control sample, antisera (diluted with washing buffer with 0.5% BSA) were added, in triplicate, to the ELISA plate at 100 μl/well. The plates were incubated for 1 hr at 37°C. The plates were then washed 3 times, followed by the addition of 100 μl detecting 2nd antibody (sheep anti-mouse IgG-horseradish peroxidase conjugate or sheep anti-mouse IgE) and incubated for 1 hr at 37°C. After this incubation period, the plates were washed and 100 μl of enzyme substrate was added to each well and the plates were protected from light. For those plates of detecting IgE, another 1 hr incubation period with a 3rd antibody, donkey anti-sheep IgG-horseradish peroxidase conjugate was needed before adding the substrate solution. The substrate solution was o-phenylenediamine dihydrochloride (OPD). OPD tablets (4 mg/tablet) were dissolved in substrate buffer (0.2 M phosphate-citrate buffer, pH 5.0) containing 0.04% H_2O_2. This solution was always prepared and used within 60 min and held at room temperature in dark. After 30 min, the reaction was stopped with the addition of 50

μl 2.5 M H_2SO_4 and the absorbance was read on an ELISA plate reader at 490 nm.

Chapter 3: Development of Radioimmunoassay

In order to study the pharmacokinetic, biological and immunological actions of TCS and DX-TCS, a prerequisite is to develop a method capable of detecting TCS both specifically and quantitatively. Such method should be able to measure TCS in range of nanomole (10^{-9} mole) or even picomole (10^{-12} mole) in biological fluids. As a result, the advantages of radioimmunoassay (RIA), including its extreme sensitivity, simplicity and specificity, make this the method of choice for the measurement of TCS. There are generally three basic steps in performing a radioimmunoassay. They are (1) production of antiserum to the compound to be measured, (2) preparation of radioactively labelled form of the compound and (3) competitive binding assay between the labelled and unlabelled antigen to the antibodies. Since protein is usually immunogenic in its own right, production of antiserum is done by injecting the pure compound with adjuvant into a suitable animal like a rabbit. This is then followed by several booster injections. The dilution of antiserum to be used for a particular radioimmunoassay is determined by incubating a fixed concentration of labelled tracer with increasing dilutions of antiserum.

Afterwards, a suitable radioisotope should be chosen and gamma-emitting radionuclides is usually used in the preparation of labelled tracers for radioimmunoassay. ^{125}Iodine is used for the preparation of labelled protein tracers

because it has a relatively long half-life of 60 days. It is also convenient to use as it can be readily substituted into tyrosine residues of the protein by the chloramine-T method (78). The chloramine-T method is one of the widely used method of iodination. Chloramine-T is the sodium salt of the N-monochloro derivative of p-toluene sulphonamide and is a mild oxidizing agent in aqueous solution. The reactions thus involve the oxidation of Na^{125}-I in the presence of a protein containing a tyrosine residue, with the subsequent incorporation of the radioiodine into the tyrosine. The excess chloramine-T is reduced by the addition of sodium metabisulphite, and free iodine is reduced to iodide. After the preparation of labelled antigen, competitive binding radioimmunoassay can then be performed.

Method

The full experimental details of production of antiserum, iodination of TCS and the radioimmunoassay procedure were described in methodology (Chapter 2). Since the assay developed will be used to detect TCS in plasma and urine, the effects of adding normal rat plasma and urine in the standard assay tubes was done. Its aim is to examine the specificity of the assay and to see if there is any constituent in the plasma or urine that might interfere with the antigen-antibody reaction.

To determine the inherent variability within a single assay, ten replicate tubes containing known quantities of TCS (100 ng/ml) were measured in a single assay and the intra-assay variance was calculated. Moreover, the inherent variability between assays was also determined using aliquots of TCS solution (100 ng/ml) stored in -20°C. Two such aliquots were then included in five separate assays and the inter-assay variance was determined on this basis. Sensitivity of the assay was determined by taking the lowest TCS concentration which will give a percentage binding that can be distinguished from zero binding ($\geq$ 2 S.D.).

Statistical Analysis

Statistical analysis between the standard curves done in buffer, plasma and urine was performed by using 10 randomly picked standard curves of each kind. Their slopes and Y-intercepts were compared by one way analysis of variance followed by Scheffe's multiple comparison test using the SPSS-PC+ computer program. The curves were considered identical if their slopes and intercepts did not different significantly ($p < 0.05$).

Results

Fig. 3.1 shows the gel-filtration profile of radioiodinated TCS. The ^{125}I-TCS

which had a higher molecular weight than free ^{125}I, was eluted out first. The tubes that contained the highest count were used in the radioimmunoassay. The % of iodine incorporated was calculated as about 51% and the specific activity of TCS was over 50 μCi/μg (1300 Ci/mmol).

Fig. 3.2 shows the typical standard curves obtained from the radioimmunoassay. Calibration curves were constructed using the coordinates B/B0 X 100% versus the logarithm of the unlabelled antigen concentration. Under the conditions stated above, the specific binding (B0) was about 33% while the non-specific binding was less than 3%. When comparing the three curves, addition of normal rat plasma makes no statistical difference while addition of rat urine shifted the curve to the right. The intra-assay and inter-assay variance were 7.5% and 9.7% respectively. The sensitivity of the assay was found to be 2.5 ng/ml in plasma and 5.0 ng/ml in urine.

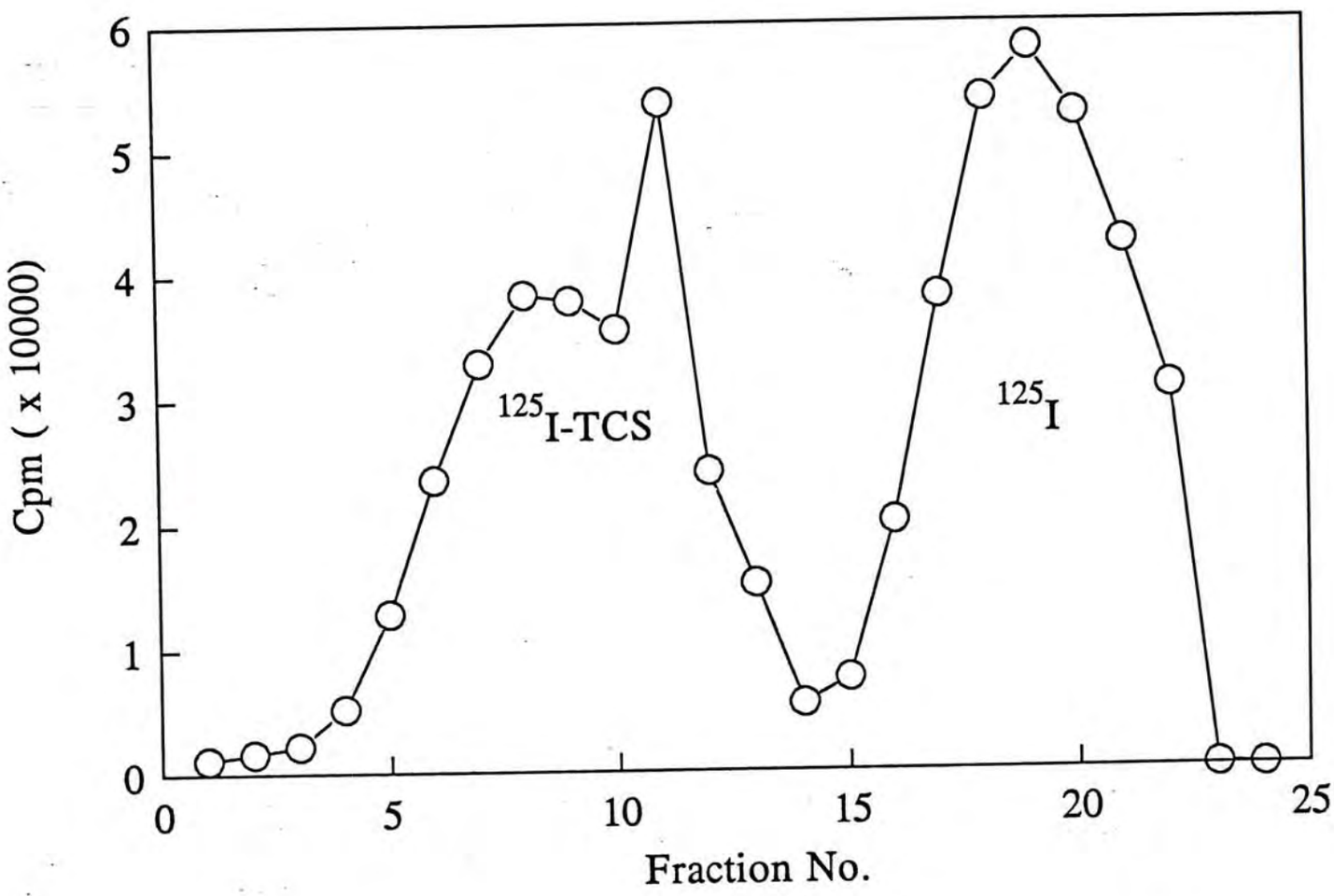


Fig. 3.1 Chromatogram on the separation of radioiodinated TCS (^{125}I-TCS) from free ^{125}Iodine (^{125}I).

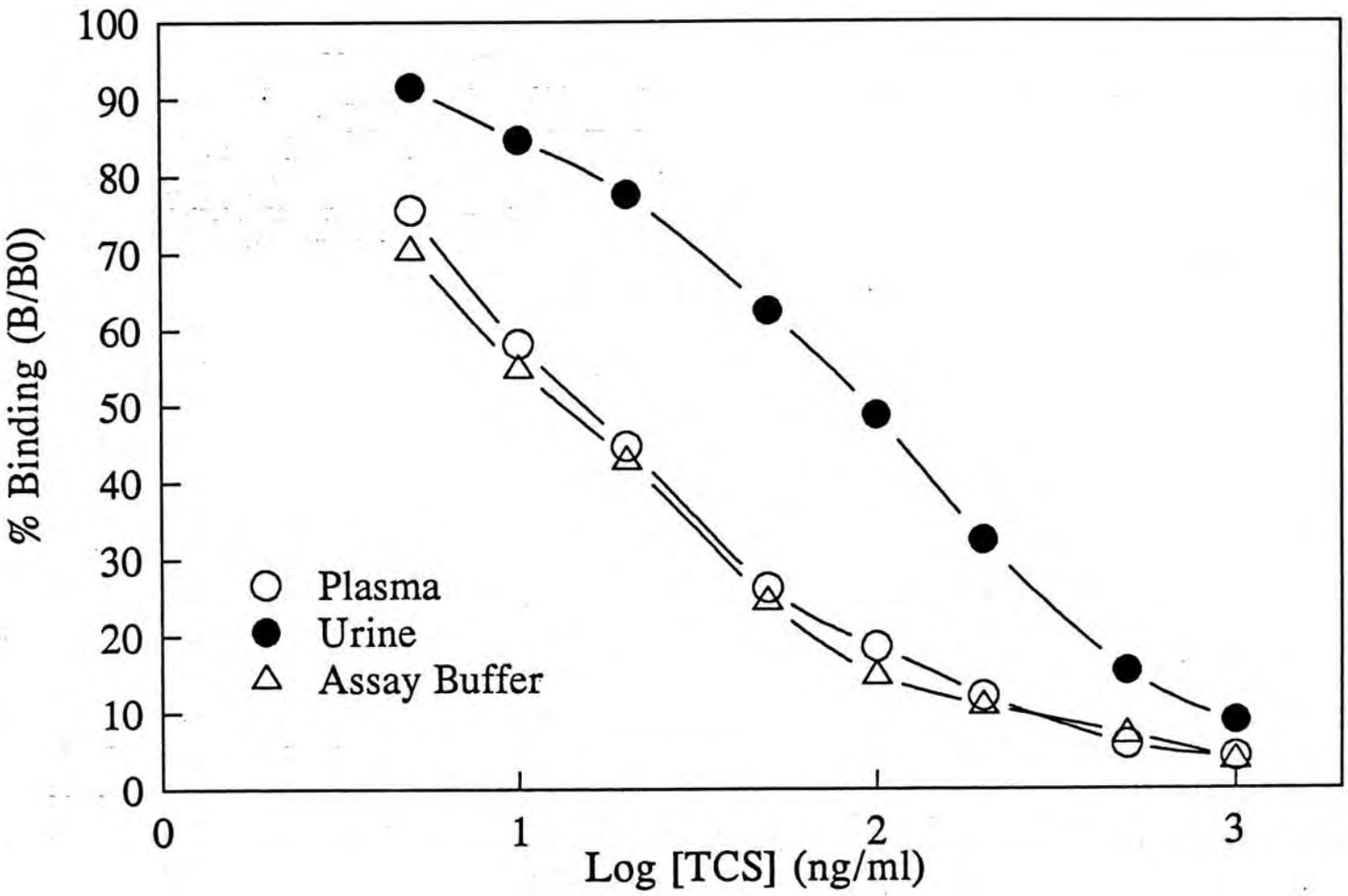


Fig. 3.2 Typical standard curves for TCS assay done in buffer, plasma and urine.

Discussion

The chloramine-T reaction during iodination seems satisfactory since high incorporation of radioiodine into protein was obtained (Fig.3.1). In fact, this method is technically simple and rapid to perform and is usually used to iodinate a protein in the first time. It is then used routinely to iodinate TCS for use as tracer in the subsequent radioimmunoassays.

A sensitive radioimmunoassay was successfully developed to detect TCS both specifically and quantitatively. The assay conditions were also optimum since we could obtain about 30% specific binding (B0) which would consequently yield a better sensitivity of the assay. Indeed, it is rather sensitive since it can detect TCS in the nanogram range. Moreover, the non-specific binding was insignificant (only 3%). Addition of 10% polyethylene glycol increased the size of the precipitate through the decrease in solubility of immune complexes without affecting the non-specific binding. The assay also appears to be reasonably consistent in the measurement of TCS according to the calculated coefficients of variance.

The assay performed in rat plasma was not different from those in standard assay buffer showing minimal interference by plasma components in the measurement. However, there was a right shift of the standard curve after addition

of urine in the assay. This may be due to some inhibitory substances present in the urine that affect the antibody-antigen reaction. Girard *et al.* found that non-specific factors in urine, measuring as apparent growth hormone, were detected when the radioimmunoassay for plasma growth hormone was applied to urine. The factors was identified as salt (NaCl) and urea presented in the urine (97). Srivastava *et al.* also proved this inhibitory effect by showing the shifting of standard curve to the right after addition of urea or NaCl (98). This phenomena is termed as "non-specific non-specificity" which refers to the interference by materials which do not directly react with the antibody, but can nevertheless affect the primary antibody-antigen reaction. For this reason, we should eliminate the "matrix effects" of urine when using RIA to measure samples. The best approach is to ensure the composition of the standards mimics that of the unknown samples. This can be done by adding normal rat urine to the standard tubes so that a urine standard curve is obtained for the measurement of TCS in urine.

Chapter 4: Coupling of TCS to Dextran T40

The molecular weight of TCS is only 26 kDa and is therefore small enough to undergo glomerular filtration. This would lead to a short half-life of this protein in the body due to rapid renal clearance (50,51). As a foreign protein, it is also immunogenic in nature as it would cause anaphylactic response in some patients receiving the drug (93,94). These problems may be overcome by modification of the protein. Thus, an appealing solution is to couple TCS covalently to another macromolecule like dextran T40 (average molecular weight of 40 kDa). There are two potential consequences after coupling. First, it increases the molecular weight of TCS from 26 kDa to 66 kDa. If glomerular filtration is an important determinant of TCS elimination, filtration of the larger TCS through the kidneys will be greatly impaired and its serum half-life can be prolonged. Secondly, the immunogenicity of TCS may be decreased when complexed with dextran. The binding of the polysaccharide sterically protects the foreign protein from recognition by the immune system through the blocking of the epitopes on the molecule.

Dialdehyde method was used for the coupling of TCS to dextran. This method had been adopted by Tam *et al* (66) for conjugation of haemoglobin to dextran. In fact, it was first developed by Flemming *et al* (81) for coupling trypsin to the insoluble cellulose. In this method, the adjacent hydroxyl groups of dextran are first

oxidized by sodium periodate and the ring structure of dextran is opened to form a dialdehyde function which in turn reacted with the amino group of TCS through the carbinolamine linkage (Fig4.1). Successful coupling was then confirmed by biochemical methods such as gel-filtration chromatography or SDS polyacrylamide gel electrophoresis.

Method

The method of coupling used by Tam (66) was generally followed and described in methodology (Chapter 2). During coupling, various TCS to dextran molar ratios (1:2.5, 1:5, 1:10, 1:25, 1:50, 1:100) were done. The purpose is to obtain an optimum TCS to dextran molar ratio in the coupling reaction.

After the reaction, the efficiency of coupling was examined. If complete coupling occurred, there was no need to separate the free TCS from the conjugated TCS. Apart from gel-filtration chromatography as described previously, successful coupling was also confirmed by using Centricon-30 microconcentrator. The DX-TCS solution prepared was loaded to the concentrator's sample reservoir and the device was centrifuged by a fixed angle centrifuge. Centrifugal force drives solvents and the low molecular weight solutes through the membrane with a molecular weight cut-off of 30 kDa, while macromolecules above the membrane cut-off are retained in the

sample reservoir. The contents that were filtered through the membrane were then analyzed by radioimmunoassay. Trichosanthin with a molecular weight below 30,000 would pass through the membrane and appear in the filtrate but not DX-TCS.

The approximate molecular weight of the conjugate was also determined by SDS polyacrylamide gel electrophoresis by comparing with SDS molecular weight markers. The polyacrylamide gel was stained using silver staining kit (Sigma) with the detection limit between 10 to 50 nanogram of protein. Stability of the conjugate after storage for different period of time (up to 6 months) at 4°C was also examined by the above method. In addition, the UV absorption spectrum before and after coupling was examined using the scanning spectrophotometer with the range from O.D. 190 to O.D. 300 nm.

Result

Fig. 4.2 shows the elution profile of different reaction mixtures at various dextran and TCS molar ratios on a Sephadex G-200 column. TCS was eluted out as a single peak at Fraction No. 23-24 (Fig. 4.2A). After the coupling reaction, TCS activity was eluted out at a much faster rate on the Sephadex column. This indicated successful coupling since the DX-TCS complex now had a larger molecular size and moved faster during gel filtration chromatography. Moreover, at high ratio of TCS

to dextran (over 1:25), coupling was essentially complete and there was clear separation between TCS and DX-TCS (Fig. 4.2B). In addition, when TCS to dextran ratio was less than 1:25, coupling became incomplete as indicated by the presence of unreacted TCS in the position of TCS peak (Fig. 4.2A). Preparation of DX-TCS for all subsequent experiments was therefore done at TCS to dextran molar ratio of 1:25 which is the lowest molar ratio producing complete coupling.

Besides, ultrafiltration study through a Centricon-30 (molecular cut off 30,000) microconcentrator showed that TCS was able to pass through the membrane while DX-TCS (1:25) could not since no detectable TCS activity was found in the filtrate. This further supported that coupling of TCS to dextran was complete.

Similar pattern was obtained in SDS polyacrylamide gel electrophoresis, free TCS was detected when the ratio of TCS and dextran was less than 1:25 while complete coupling was seen when the ratio was above 1:25. There was a continuous distribution of the molecular size of DX-TCS (Fig. 4.3). Molecular weight was over 66 kDa as determined from the molecular weight markers. Indeed, similar pattern was observed in gel filtration chromatograph as DX-TCS was eluted out near the void volume. The conjugate seemed to be very stable. No observable change occurred when stored at 4°C for up to six months as seen from the result of polyacrylamide gel electrophoresis. On the other hand, coupling produced minimal change to the UV

absorption spectrum of the protein (Fig. 4.4) especially at O.D. 280 nm which was used for the determination of protein concentration.

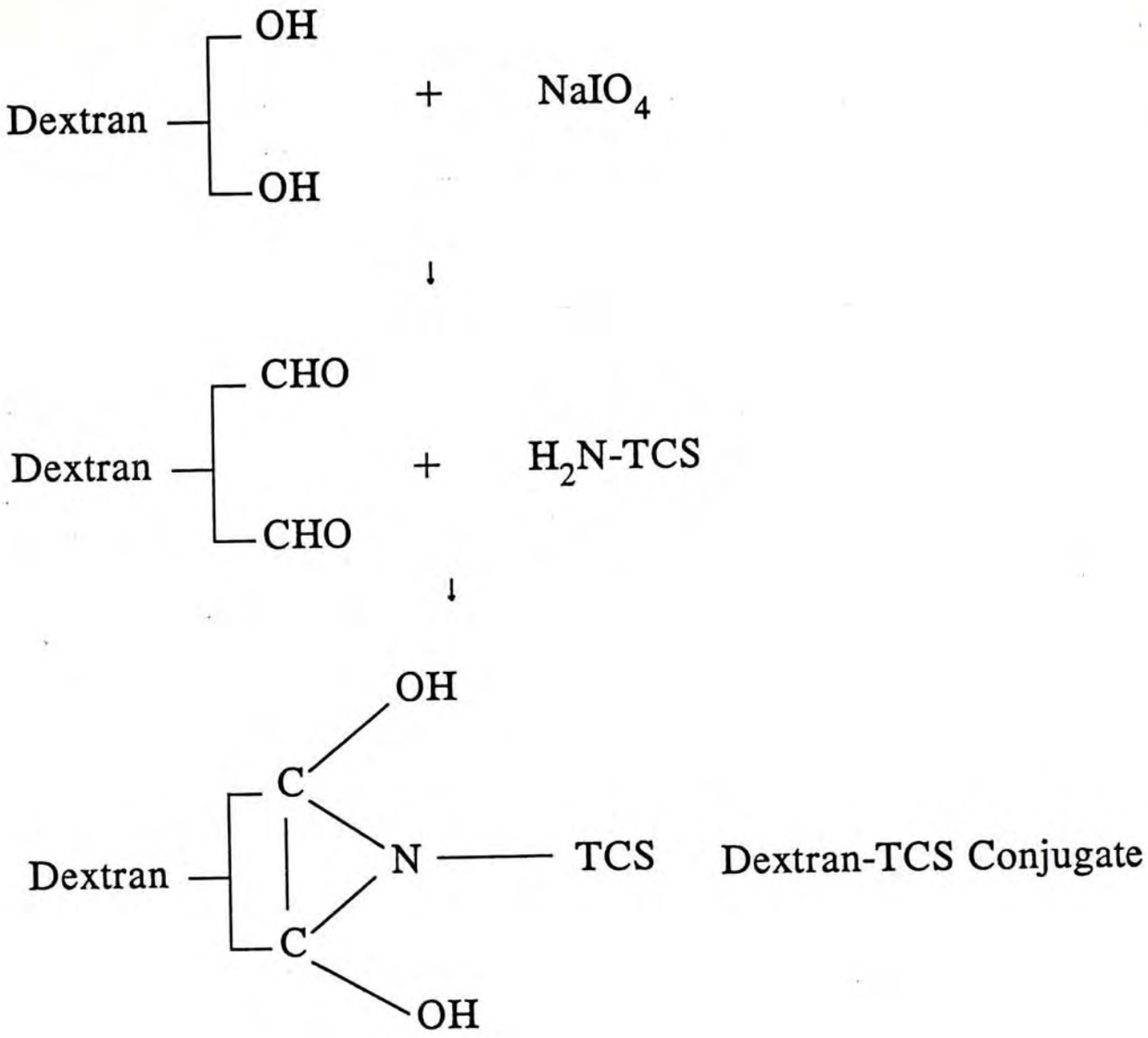
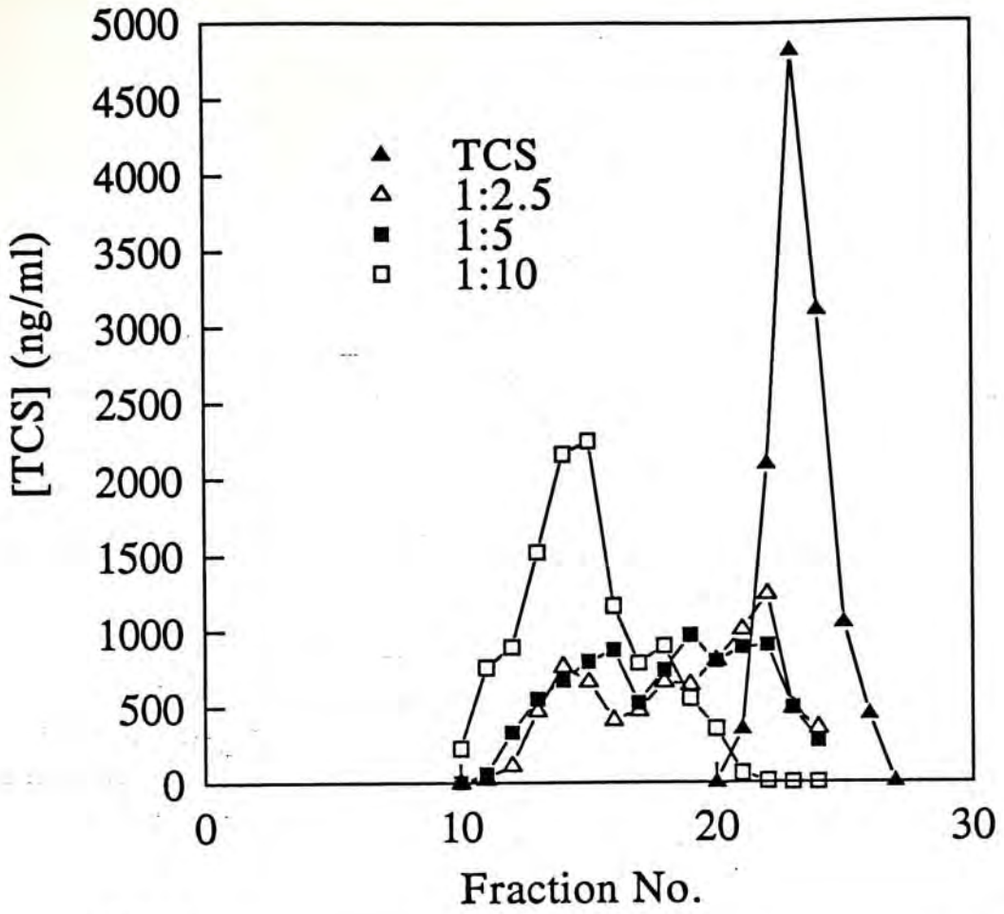


Fig. 4.1 Coupling of Trichosanthin to dextran by dialdehyde method.



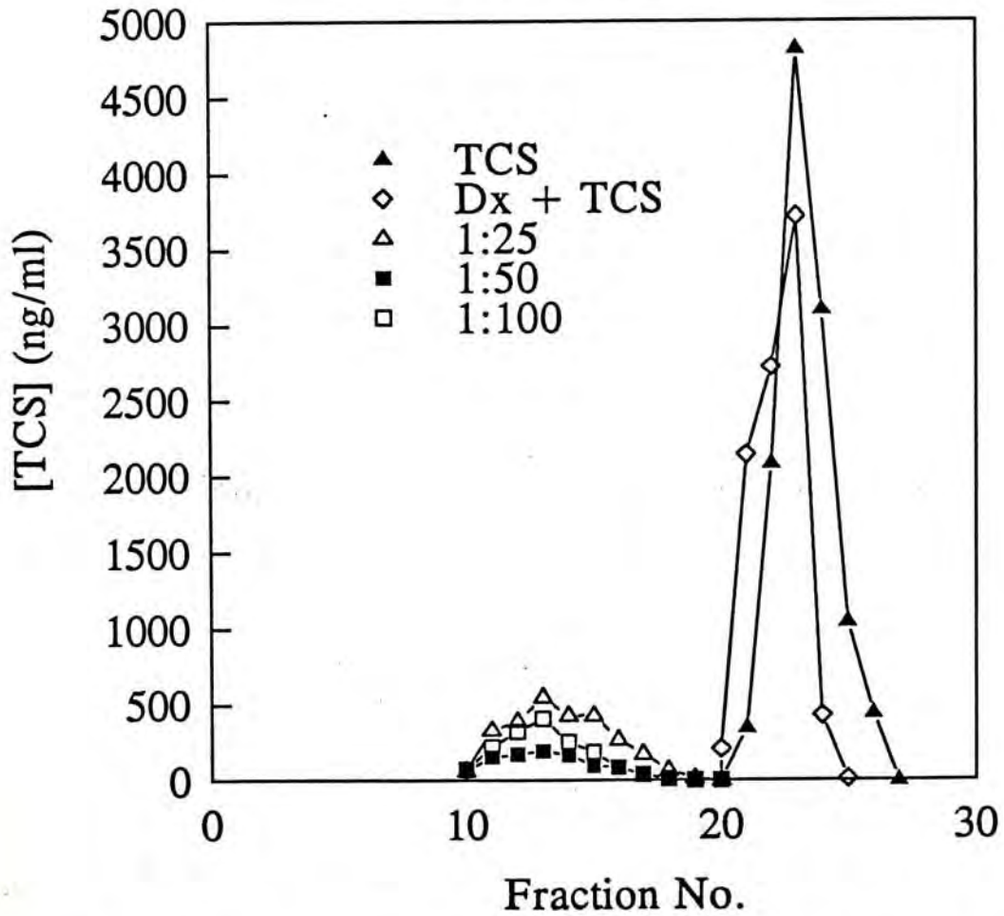


Fig. 4.2 Elution profile of different reaction mixtures at different dextran and TCS ratios on Sephadex G-200 column.

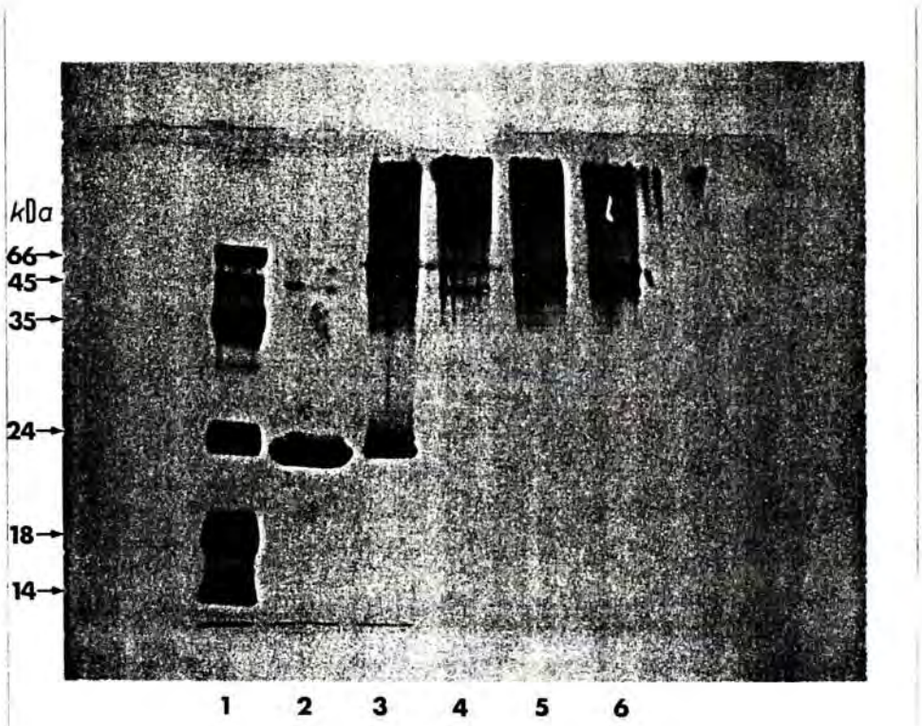


Fig. 4.3 SDS PAGE electrophoresis of TCS and DX-TCS. Lane 1: molecular mass standards; Lane 2: TCS; Lane 3: DX-TCS with TCS to dextran molar ratio below 1:25; Lane 4-6: DX-TCS stored at 4°C for 1 month, 3 months and 6 months respectively.

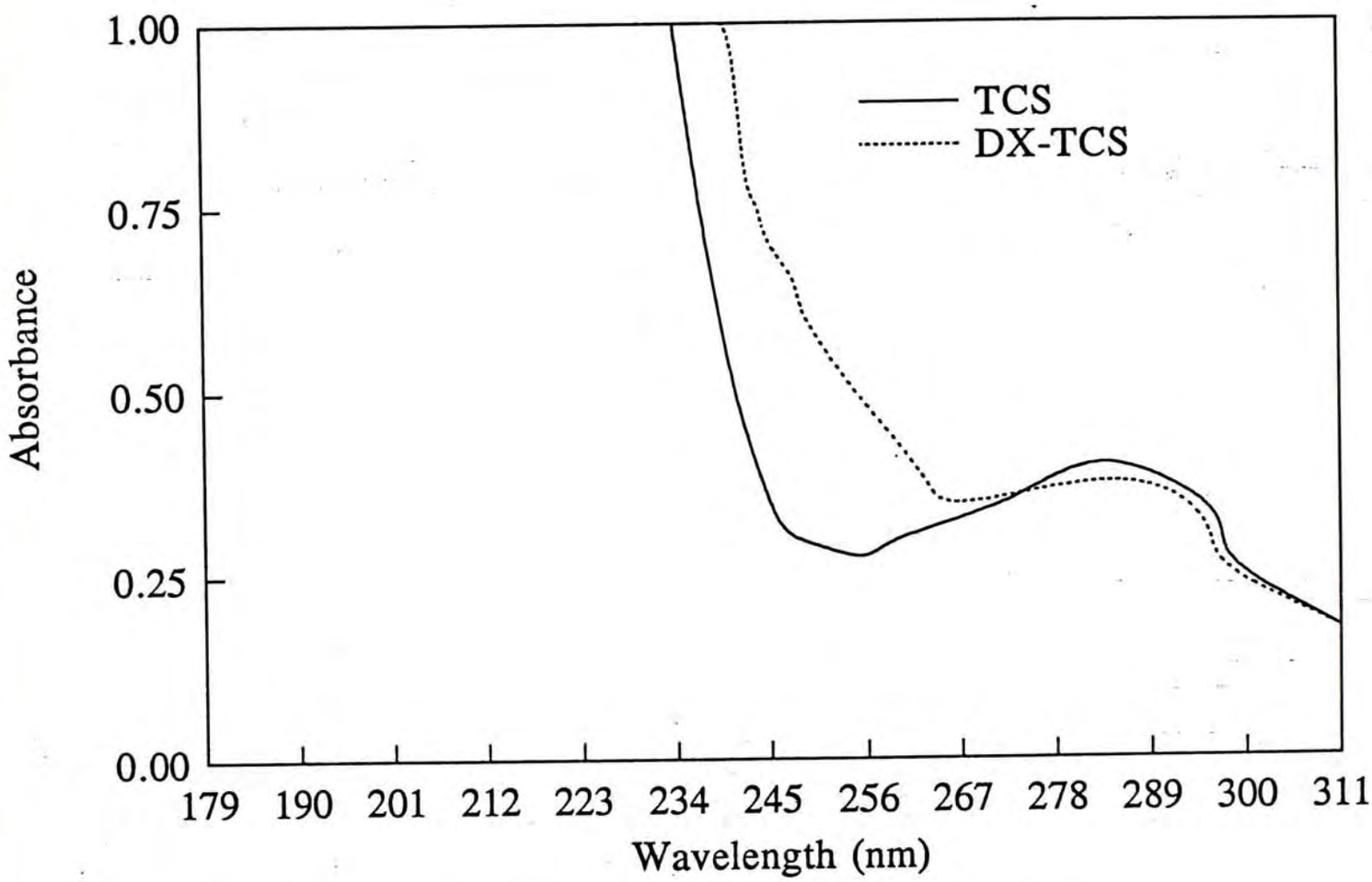


Fig. 4.4 Scanning spectra of TCS and DX-TCS at the UV region.

Discussion

Dialdehyde method is one of the most common procedure developed for coupling proteins to dextran. It is rapid, simple, straightforward and safe. Indeed, many examples of dextran periodate activation for the preparation of protein conjugates are described in the literature (61,62,71,72). During conjugation, oxidation of the hydroxyls of dextran with sodium periodate solutions leads to aldehyde functionalities that easily react with amine derivatives (82). It should be reminded that the activated dextran or polyaldehyde dextran has to be used in a short period of time (about one week) for the conjugation reaction. The addition of sodium bisulphite solution serves to reduce the unreacted periodate and prevent further formation of dialdehyde.

Different molar ratio of dextran to TCS was used in the coupling reaction. Its aim is to find an optimum reaction condition so that the yield of synthesis of the complex could approach completion. As a result, separation of DX-TCS from residual TCS could be avoided. Complete coupling was seen when the molar ratio of TCS to dextran was 1:25 due to the abundance of activated dextran to bind the TCS molecule. Hence, it was unnecessary to use a higher molar excess of dextran over TCS to achieve completion. However, a molar ratio below 1:25 was insufficient to drive the reaction towards completion and free TCS remained.

The oxidized dextrans are multifunctional with many reactive aldehyde groups per molecule and the protein also has many reactive amino groups. Therefore, in principle, the coupling between these two types of molecules could yield a complex mixture of products differing in their molecular sizes and compositions and in their sites of coupling. In fact, this was seen in the DX-TCS preparation which showed a spectrum of molecular sizes of the conjugates ranging from over 66 kDa to near 100 kDa. Molecular weight of 66 kDa was obtained when one molecule of TCS linked to one molecule of dextran (DX_1-TCS_1). However, for higher molecular weight conjugate, there may be a chance of getting 2 TCS molecule attached to 1 dextran molecule (DX_1-TCS_2) or 1 TCS molecule attached to 2 dextran molecule (DX_2-TCS). Further increasing the size of conjugate was limited by the steric packing of dextran and TCS molecule. The conjugate was also very stable upon storage at 4°C and no free TCS was detected. Moreover, it seemed that the coupling reaction did not alter much the spectral property of the protein.

In summary, a covalent dextran-trichosanthin complex was successfully synthesized by the dialdehyde method. The reaction is reproducible with the production of a stable conjugate.

Chapter 5: Pharmacokinetic Study

After the development of a sensitive radioimmunoassay for the detection of TCS in plasma and urine and the successful coupling of TCS to dextran T40, it comes to the pharmacokinetic study of TCS and its conjugate using rat as the animal model. The aim is to evaluate the role of kidney in the elimination of the drug and to see whether coupling will increase the persistence of TCS in the circulation. If renal excretion is the major route of TCS elimination, coupling of TCS to dextran would probably prolong its half-life in the circulation by preventing it from entering the kidney. One of the examples is the coupling of haemoglobin to dextran which subsequently lead to a prolonged circulatory half-life (66). The pharmacokinetic behaviour of DX-TCS was therefore compared with that of parent compound to see how coupling affected the elimination of TCS. After the experiments, two approaches, namely (i) compartmental analysis and (ii) non-compartmental analysis were employed to characterize the pharmacokinetics of the drug and its conjugate (83).

Compartmental analysis is the most commonly employed method. The body is considered to be consist of several compartments (compartment 1, compartment 2, etc.). In a pharmacokinetic sense, a compartment refers to those organs and tissues for which the rates of uptake and subsequently clearance of drug are similar.

Sometimes these compartments have no physiologic or anatomic reality. It also assumes that the rate of transfer between compartments and the rate of drug elimination from compartments follow first-order or linear kinetics. In general, compartment 1 is the central vascular compartment whereas compartment 2 is a rapid accessible compartment (e.g kidney, liver), and compartment 3 refers to the more "deep" peripheral or slow accessible compartment like skin, bone, and muscle. During analysis, the goal is to find out whether the plasma concentration-time curves are best fitted in terms of a triexponential function (3-compartment model) or a biexponential function (2-compartment model). Their respective equations are shown below:

Triexponential equation: $Cp(t) = Ae^{-\alpha t} + B^{-ßt} + Ce^{-\tau t}$

Biexponential equation: $Cp(t) = Ae^{-\alpha t} + B^{-ßt}$

In these equations, Cp(t) is the plasma concentrations of the drug at any time (t) after i.v. drug administration. A, B, C are the corresponding zero-time intercept, and α, ß, τ are the apparent first order disposition constants.

Apart from the above method, the data were also analyzed by using the non-compartmental analysis. In this method, it does not require the assumption of specific compartmentalization and therefore can apply to virtually any compartmental model provide that we can assume linear pharmacokinetics. Both compartmental analysis and non-compartmental analysis will be used in data analysis and to see whether there are

differences between the two.

Method

The experimental protocol of the pharmacokinetic study was described in methodology. The pharmacokinetic study of TCS was done in normal rats (group 1) and renal artery-ligated rats (group 2). The pharmacokinetic behaviour of DX-TCS was examined in normal rats (group 3). The reason for doing group 2 rats is to investigate the role of kidney in eliminating TCS. Renal function of group 2 rats were impaired by bilateral ligation of the renal arteries. As a consequence, we can investigate the disposition and elimination of TCS in the absence of kidney. Tritiated inulin were administrated at the beginning of the experiment so as to access renal function. Since kidney is the only organ that removes inulin, theoretically, plasma inulin concentration should decrease with time in normal rats with normal renal function. In contrast, a constant plasma inulin concentration is expected in renal arteries ligated rats.

The plasma and urine concentrations of TCS or DX-TCS were determined by the radioimmunoassay method as described above. Determination of plasma DX-TCS was done under disequilibrium condition using DX-TCS as standards to increase the sensitivity of the assay. Thus, the unlabelled standard DX-TCS have an advantage of

binding with the TCS antibodies first at 4°C overnight. Afterwards, labelled antigen was added to react with the TCS antibodies for 24 hr at 4°C which was then followed by normal radioimmunoassay procedure.

In a separate experiment, the plasma protein binding to TCS was evaluated as mentioned in the methodology (Chapter 2). In principle, any molecule with a molecular weight below 50,000 and not bound to any plasma protein are filterable through the kidney. Therefore, in addition to the pharmacokinetic study, it is important to demonstrate the plasma protein binding of TCS is minimal in order to support that it is filtered through the kidney.

Statistical analysis

Pharmacokinetic parameters were calculated by the BITRI computer program using method of residuals for compartmental analysis (83) and the PKCALC computer program for non-compartmental analysis. Statistical analysis of group differences were performed by Tamhane's multiple comparison with unequal variances (99) at significant level of $p < 0.05$.

Result

After a single bolus injection of tritiated inulin, a constant plasma inulin concentration was observed in the ligated rats (group 2) demonstrating that there was a complete loss of renal function in this group of rats (Fig. 5.1). On the other hand, the plasma concentration of inulin in normal rats (group 1) decreased with time. As a consequence, the injected TCS in group 2 rats was now distributed and eliminated without the involvement of kidneys when compared with group 1 rats. Blood pressure was not changed after injection of TCS or DX-TCS into the rats. There was also swelling of the footpads of the rat receiving DX-TCS injection.

Figure 5.2 shows the plasma concentration vs time in three groups of rats. When TCS was injected into normal rats, the concentration of TCS declined rapidly within 45 min and resulted in a 100 fold difference from time zero. In contrast, when TCS was injected into ligated rats, the plasma concentration of TCS decreased in a much slower rate. Injection of DX-TCS resulted in a similar pattern as in the ligated rats. The plasma DX-TCS concentration was highest at any time during the experimental period comparing to the injection of same dose of TCS (in both normal and ligated rats). Some calculated pharmacokinetic parameters were summarized in Table 5.3 using non-compartmental analysis. Comparing with TCS injected into normal and ligated rats, DX-TCS injection resulted in having the longest mean

residence time (MRT) which represents the time for about 60% of the administered dose to be eliminated. Area under curve (AUC) was also found to be the highest which indicates a change in total clearance rate. In fact, this can be seen as the plasma clearance (Cl_p) was lowest for DX-TCS. All these parameters suggested that the modified form of TCS stayed much longer in the circulation.

When using compartmental analysis, most of the pharmacokinetic parameters calculated (e.g. AUC, Cl_p, etc.) were similar to the non-compartmental analysis and followed the same trend (Table 5.4). In addition, the $t_{1/2}(\alpha)$, which is the half-life of the drug at the initial distribution phase, was longest in ligated rats. Moreover, TCS injection (group 1) was consistent with a three-compartment model while DX-TCS injection (group 3) or TCS injection into ligated rats (group 2) were consistent with a two-compartment model. All pharmacokinetic parameters (Table 5.3 and 5.4) were significantly different between the three groups of rats ($p < 0.05$).

Urinary excretion data showed that only $0.38 \pm 0.05\%$ of the administered TCS was recovered in urine within 180 min of experimental period. Nearly all the urine TCS was recovered in the first 45 min interval ($86 \pm 5\%$). No DX-TCS activity was detected in the rats with DX-TCS injection (group 3).

In the plasma protein binding experiment, result demonstrated that plasma

protein binding to TCS was minimal. Incubation of TCS with plasma did not affect its ultrafiltration in the Centricon-30 microconcentrator. The filtrate concentration of TCS incubated with PBS and plasma was 269 $\pm$ 54 and 284 $\pm$ 62 ng/ml respectively.

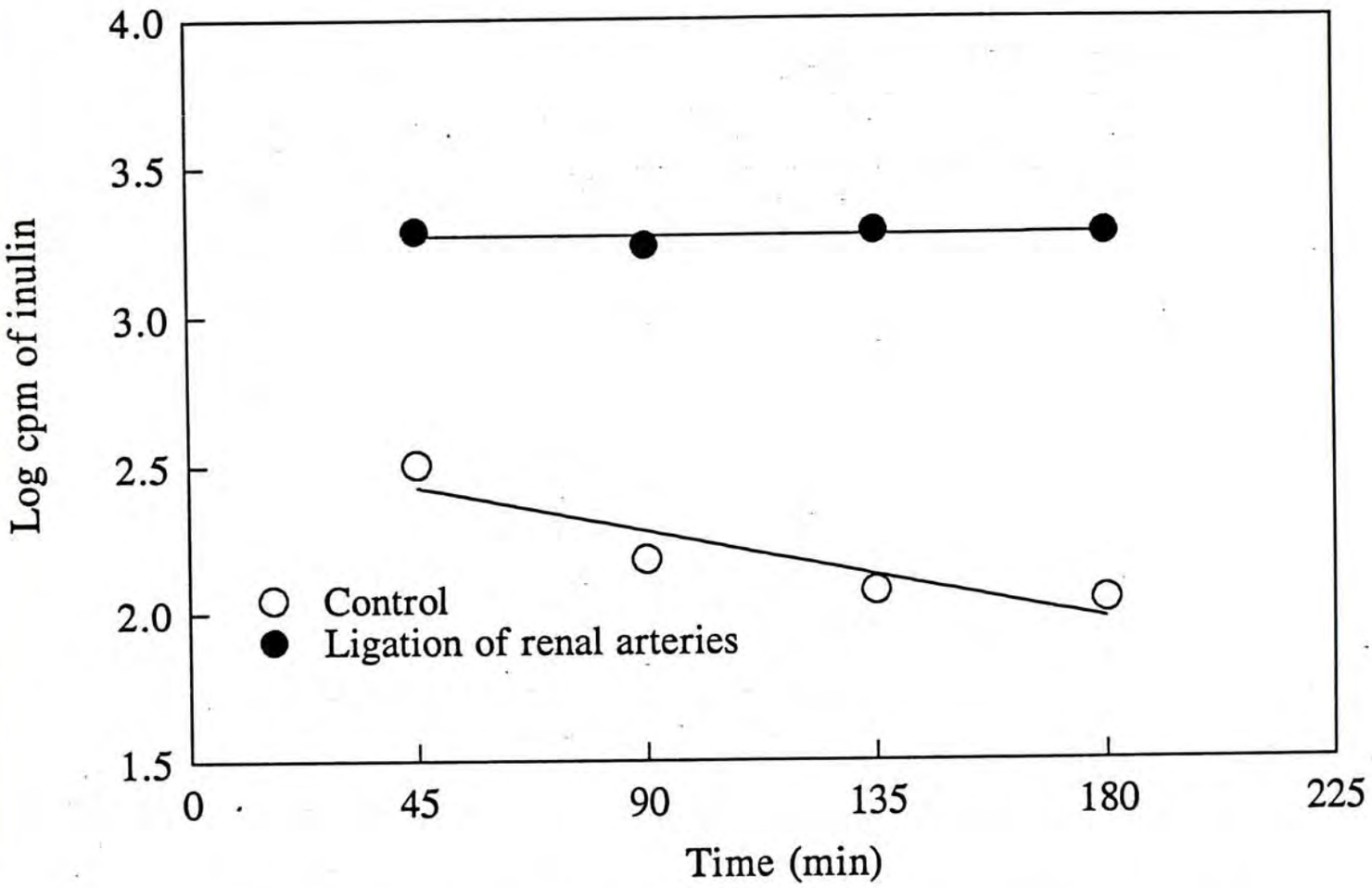


Fig. 5.1 Plasma inulin concentration in normal and ligated rats. Values are expressed as means ± SEM (N=6).

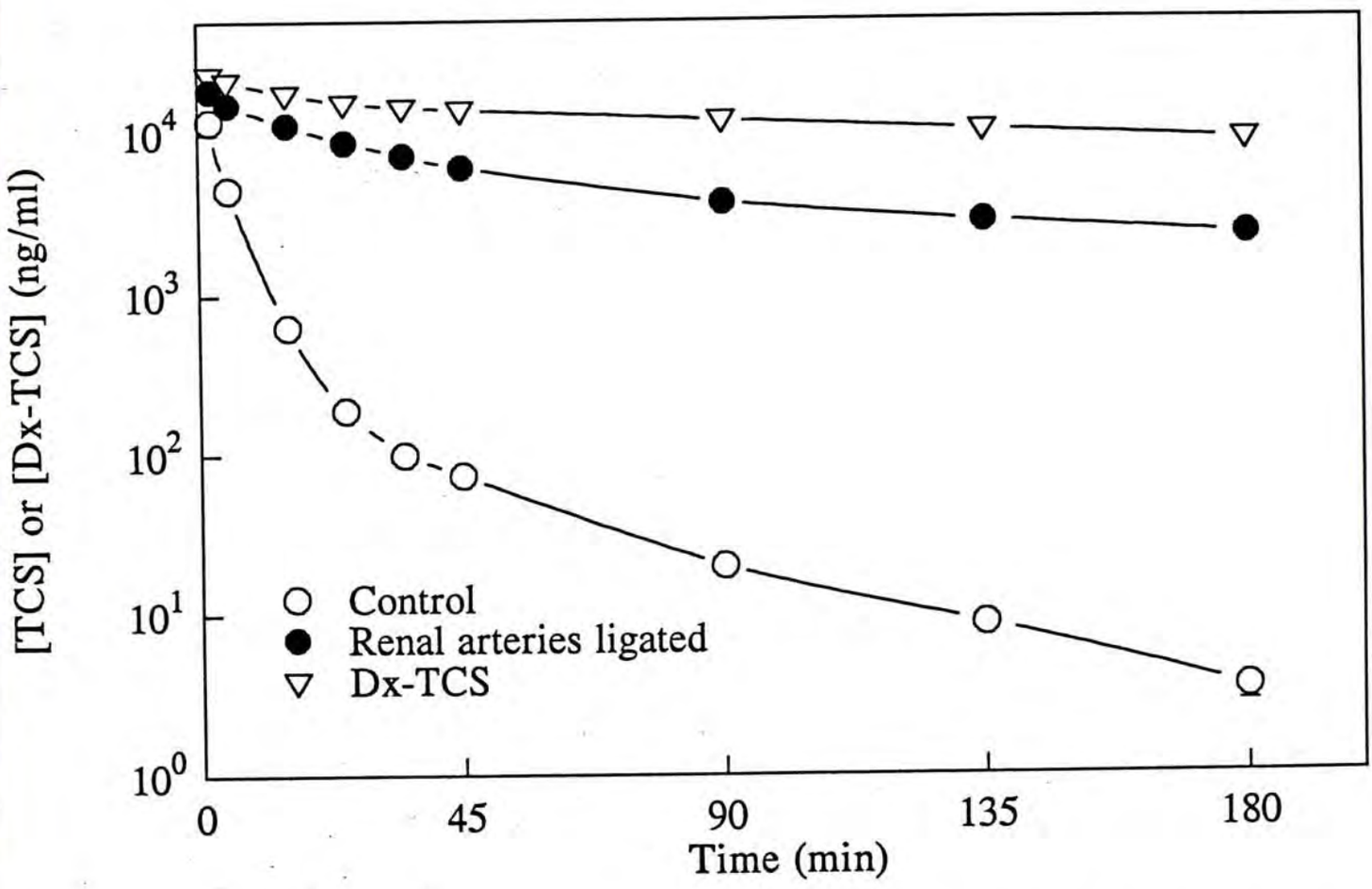


Fig. 5.2 Plasma concentration time curves of 0.75 mg/kg TCS injection into normal (N=6) and ligated (N=8) rats, and DX-TCS injection (0.75 mg/kg) into normal rats (N=6). Values are expressed as menas ± SEM.

	Normal (N=6)	Ligated (N=8)	DX-TCS (N=6)
$t_{1/2}(\alpha)$ (min)	2.2 ± 0.3	14.5 ± 1.1	8.5 ± 0.9
V_z (ml/kg)	506 ± 40	142 ± 14	46 ± 1
V_{ss} (ml/kg)	75 ± 6	96 ± 8	45 ± 1
AUC_0^∞ (μg-min/ml)	81 ± 16	1534 ± 216	5228 ± 684
Cl_p (ml/min)	3.44 ± 0.35	0.20 ± 0.02	0.06 ± 0.01

Table 5.3 A summary of some pharmacokinetic parameters in normal rats injected with TCS or DX-TCS and ligated rats injected with TCS using compartmental analysis. Values are expressed as means ± SEM. All three groups are significantly different from each other ($p < 0.05$). Abbreviations: $t_{1/2}(\alpha)$, distribution half-life; V_z, apparent distribution volume, V_{ss}, steady state distribution volume; AUC_0^∞, area under curve; Cl_p, plasma clearance.

	Normal (N=6)	Ligated (N=8)	DX-TCS (N=6)
V_z (ml/kg)	507 ± 36	97 ± 6	45 ± 1
V_{ss} (ml/kg)	127 ± 9	84 ± 4	45 ± 1
AUC_0^∞ (μg-min/ml)	59 ± 6	1285 ± 102	5095 ± 1634
Cl_p (ml/min)	4.78 ± 0.57	0.22 ± 0.02	0.06 ± 0.04
MRT (min)	9 ± 1	145 ± 16	308 ± 41

Fig. 5.4 A summary of some pharmacokinetic parameters in normal rats injected with TCS or DX-TCS and ligated rats injected with TCS using non-compartmental analysis. Values are expressed as means ± SEM. All three groups are significantly different from each other ($p < 0.05$). Abbreviations: V_z, apparent distribution volume, V_{ss}, steady state distribution volume; AUC_0^∞, area under curve; Cl_p, plasma clearance; MRT, mean residence time.

Discussion

It is expected that TCS can be filtered into the renal tubule due to its small molecular size and non-protein binding nature. The pharmacokinetic data obtained do suggest that this is the case. In the absence of renal function, the increase in area under curve (AUC), mean residence time (MRT) and the reduction in plasma clearance (Cl_p) all suggested that kidney is the major eliminating organ for TCS.

In compartmental analysis, the half-life of the initial distribution phase ($t_{1/2}\alpha$) was increased in ligated rats meaning that in this early distribution phase, plasma TCS declined at a much slower rate in the absence of renal function. On the other hand, in normal rats, enormous amount of TCS was being distributed into the kidney followed by elimination during the initial phase. Rapid renal loss of TCS was also due to the high TCS concentration which in turn lead to an elevated filtered load. Another interesting finding in compartmental analysis is that DX-TCS injection was consistent with two-compartment model. Both V_z and V_{ss} were found to decrease. It seems that a major distribution compartment of TCS is lost after coupling to dextran. This compartment is most likely the kidney since DX-TCS conjugate cannot enter it due to its large molecular size (over 66 kDa). This is analogous to the ligated rats which do not have a functional kidney.

Injection of DX-TCS produced the highest plasma concentration at any time during the entire experimental period as seen from Fig. 5.2 and the plasma DX-TCS concentration gradually decrease with time. This suggests the existing of extra-renal degradation of the conjugate since now the larger complex was not able to enter the kidney for elimination. The exact metabolic site was still unknown. Liver and the reticulo-endothelial system are the possible candidate since dextran conjugate has been reported to be taken up and degraded by these two sites (74). On the other hand, the pharmacokinetic parameters such as MRT, AUC, Cl_p all supported that DX-TCS stayed much longer than TCS in normal rats and even in ligated rats. On the basis of these finding, if glomerular filtration of TCS is the only parameter that affects its elimination and distribution, one will anticipate that the pharmacokinetic behaviour of DX-TCS in normal rats will be the same as TCS in ligated rats. However, from both the concentration time curve and the pharmacokinetic parameters, DX-TCS remained even longer in the plasma compartment than TCS in ligated rats. This suggests that coupling of TCS to dextran not only reduces it rate of renal clearance, but also its rate of non-renal degradation. Probable, it may due to the fact that the covalent complex is more resistant to enzymatic degradation.

Rats are known to be sensitive to dextran (106). Indeed, this was demonstrated by the swelling of the footpads of the rat with DX-TCS injection. However, because there is no change in blood pressure and glomerular filtration rate (data shown in

Chapter 6), the possibility that the sensitivity might affect the pharmacokinetic observations is therefore remote.

When estimated from the mean residence time (about 10 min in normal rats), more than 60% of the administered TCS was removed within the experimental period of 180 min. As mentioned above, if kidney is the major eliminating organ of TCS, one would anticipate that large amount of TCS be found in urine. However, the total amount of TCS that could be recovered from urine within the experimental period of 180 min was only 0.38 $\pm$ 0.05% which was much lower than expected. It then led to the speculation that after glomerular filtration, a large amount of TCS was somehow retained by kidney. It may be due to the precipitation of TCS in the renal tubules, being reabsorbed by tubular cells or non-specific binding by kidney tissues so that the amount of TCS cleared by the kidney cannot be fully recovered in urine. Amongst the possibilities, reabsorption of TCS by renal tubular cells is most likely as this is a common metabolic pathway of low molecular weight proteins (74,84,85). Further study to clarify this issue was done and discussed in the later chapter.

In summary, kidney is major organ of elimination of TCS. Covalent coupling of TCS to dextran prolongs the survival of TCS in the circulation, mainly due to the increase in molecular size and thus the conjugate can escape from glomerular filtration.

Chapter 6: Renal tubular reabsorption of TCS

In the previous chapter, it was shown that kidney is the major organ that eliminates TCS. Unfortunately, the amount of TCS lost could not be recovered in urine. Indeed, urine recovery of TCS was very small. A possible explanation is that the filtered TCS is reabsorbed by the renal tubular cells, degraded in the cells and the amino acids returned to general circulation.

Kidney is an important site for the catabolism of low molecular weight proteins which have molecular weight less than 50,000 (e.g. lysozyme, insulin, ribonuclease,etc.) (74,84,85). After filtration of these proteins into the renal tubule, they are reabsorbed by receptor mediated endocytosis, and eventually catabolized in the proximal tubular cell. The entire process is rather non-specific for the nature of proteins. Trichosanthin has a molecular weight similar to these proteins and therefore it may be reabsorbed by this common endocytotic process.

In order to support the hypothesis, two sets of experiment were performed. Firstly, rats would receive escalating doses of injected TCS. The purpose is to see whether the urinary recovery of TCS can be increased by saturating the reabsorptive capacity of the tubular cells after high dosage of injection. As described beforehand, filtered low molecular weight proteins are mostly reabsorbed by renal tubular cells

through a common endocytotic process. It was demonstrated that infusion of one protein can increase the excretion of another protein (86,87). Adopting this thought, in the second set of experiment, the competitive nature of TCS was examined. Hence, filterable proteins (haemoglobin and lysozyme) were infused into the animals to see if they can compete with and increase the excretion of TCS.

Method

The general procedure for these two sets of experiment were described in Chapter 2. In the first experiment in which the dosage effect on urinary TCS recovery was studied, the five injection doses used were 0.375, 0.75, 1.5, 3 and 12 mg/kg. After TCS injection, plasma samples were collected at 45, 90 and 135 min and urine samples were collected at 45 min interval. Plasma and urine concentrations of TCS were determined by radioimmunoassay.

In the second set of experiment, the mechanism of TCS reabsorption was examined. Rats were divided into 6 groups. After a loading dose of tritiated inulin, rats were then followed by continuous infusion of different fluids containing tritiated inulin at a rate of 3.4 ml/hr so that their respective glomerular filtration rate was monitored. Group 1, 2 and 3 rats were infused with normal saline solution (N=8), 77.5 μM stroma-free haemoglobin solution (N=9) and 77.5 μM lysozyme solution

(N=9) respectively. Trichosanthin (0.375 mg/kg) was injected at 90 min after onset of infusion. Plasma and urine samples were taken at 45 min interval and the concentrations measured by radioimmunoassay. Moreover, group 4 rats (N=5) were continuously infused with normal saline solution but with DX-TCS (0.375 mg/kg) injection. Group 5 (N=7) and group 6 (N=6) rats were infused with normal saline and lysozyme solution only without any drug injection. Stroma-free haemoglobin solution was prepared according to a previously described method (66). Plasma haemoglobin concentration was measured by the method of Drabkin and Austin (89). In this method, 0.04 ml sample was pipetted into a test tube and 2.5 ml of Drabkin's solution was added. During the reaction, haemoglobin was first oxidized with potassium hexacyanoferrate (III) to methaemoglobin which then reacted with potassium cyanide to form cyanmethaemoglobin. After 10 min, the optical densities of the samples were measured by a spectrophotometer at 540 nm and the results were compared with the standard curve to find out the concentration of the samples. Glomerular filtration rate was estimated from inulin clearance. Quenching was corrected by external standard. Since the urine samples will contain haemoglobin or lysozyme. The effect of these proteins on the TCS radioimmunoassay will be examined.

Statistical analysis

Two way analysis of variance was used for comparison of urinary TCS excretion at different doses of TCS injection ($p < 0.05$). For comparing the effect of haemoglobin and lysozyme infusion on TCS excretion, one way analysis of variance followed by Scheffe multiple comparison test was used ($p < 0.05$). Multivariate analysis of variance was used for inter-group comparison of inulin clearance ($p < 0.05$).

Result

In the first experiment, urinary recovery of TCS was only $0.29 \pm 0.06\%$ when the smallest injection dose was used (0.375 mg/kg; Fig. 6.1) meaning that over 95% of the administered dose could not be found in urine although kidney is the major organ of elimination. However, the percentage of urine TCS recovery increased tremendously when higher injection dose was used. It could increase up to about 40% when 12 mg/kg TCS was injected and the curve gradually approached the plateau phase. Figure 6.2 showed the plasma concentration of TCS after different doses of drug injection. When the percentage of total TCS recovered in each 45 minute interval was calculated, the majority of the urine TCS (over 85%) was recovered in the first 45 minute after injection regardless of the doses used (Fig. 6.3). The

percentage decreased rapidly with time together with the plasma concentration of TCS. Finally only very little TCS was excreted (less than 5%) in the last 45 minute compared to the total TCS excreted.

When 77.5 μM lysozyme was infused and 0.375 mg/kg TCS was injected, the percentage of TCS recovered in urine was significantly increased from $0.29 \pm 0.06\%$ to $3.20 \pm 0.86\%$. Infusion of 77.5 μM stroma-free haemoglobin further increased the TCS recovered in urine to $35.23 \pm 2.66\%$ (Fig. 6.4). Haemoglobinuria were also observed. The plasma haemoglobin concentration was constant at about 1.0 ± 0.1 g/l.

Figure 6.5 showed the inulin clearance of different groups of rats. Injection of TCS caused a significant decrease in GFR throughout the entire experimental period. Simultaneous infusion of haemoglobin led to a further decrease in GFR. However, there was no significant change in GFR compared with the control when lysozyme was infused with TCS injection. Injection of DX-TCS or infusion with lysozyme alone also did not altered the GFR when compared with the control.

In the radioimmunoassay, addition of different concentrations of haemoglobin or lysozyme did not affect the percentage of total binding in the assay.

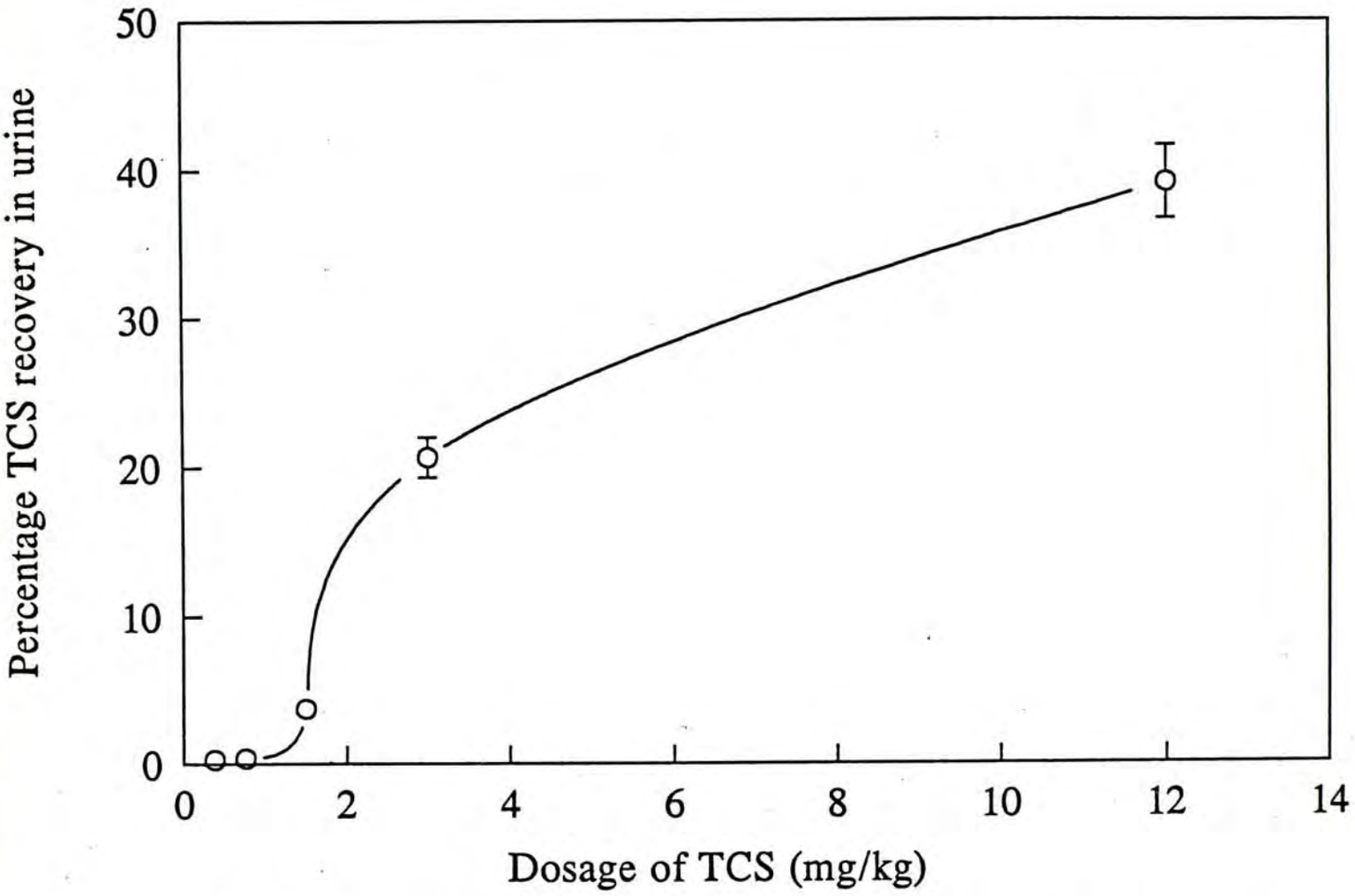


Fig.6.1 Percentage of the administered TCS recovered in urine during the 135 min period after injection. The dosage used are 0.375 (N=13), 0.75 (N=6), 1.5 (N=6), 3 (N=7), and 12 mg/kg (N=4). Values are expressed as means ± SEM.

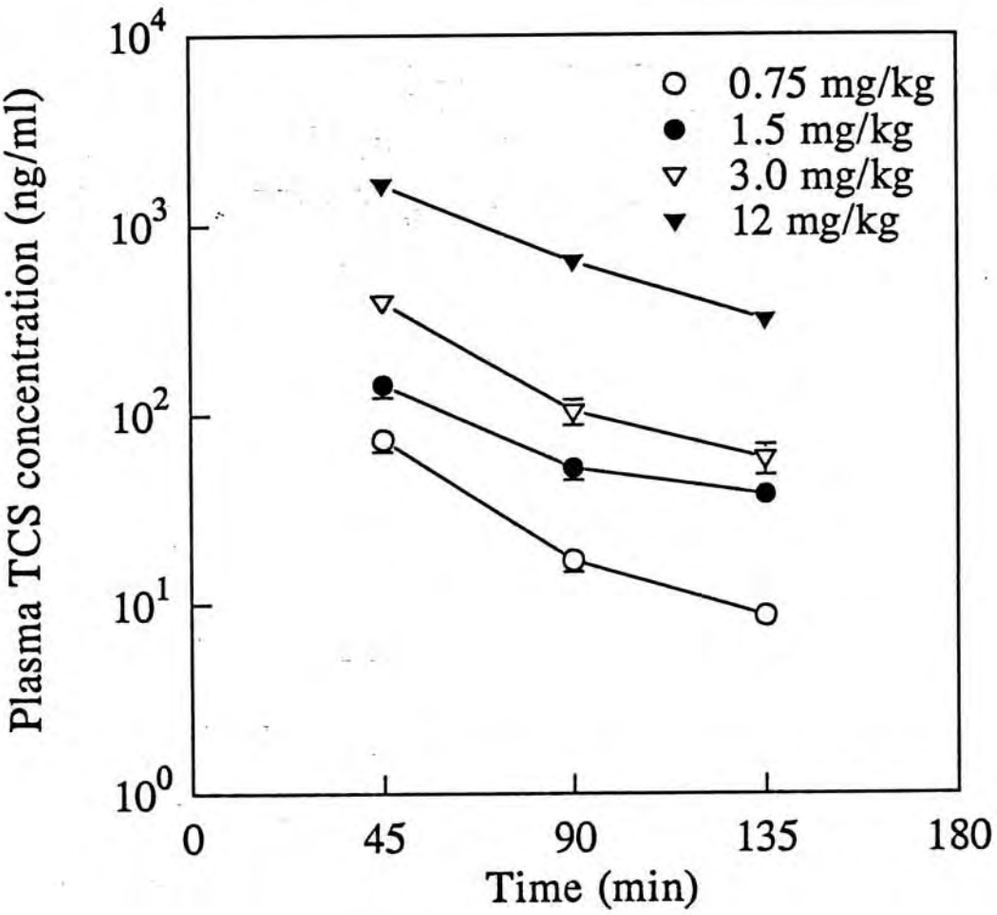


Fig. 6.2 The plasma TCS concentration at different time after injection of various doses of TCS. Values are expressed as means ± SEM.

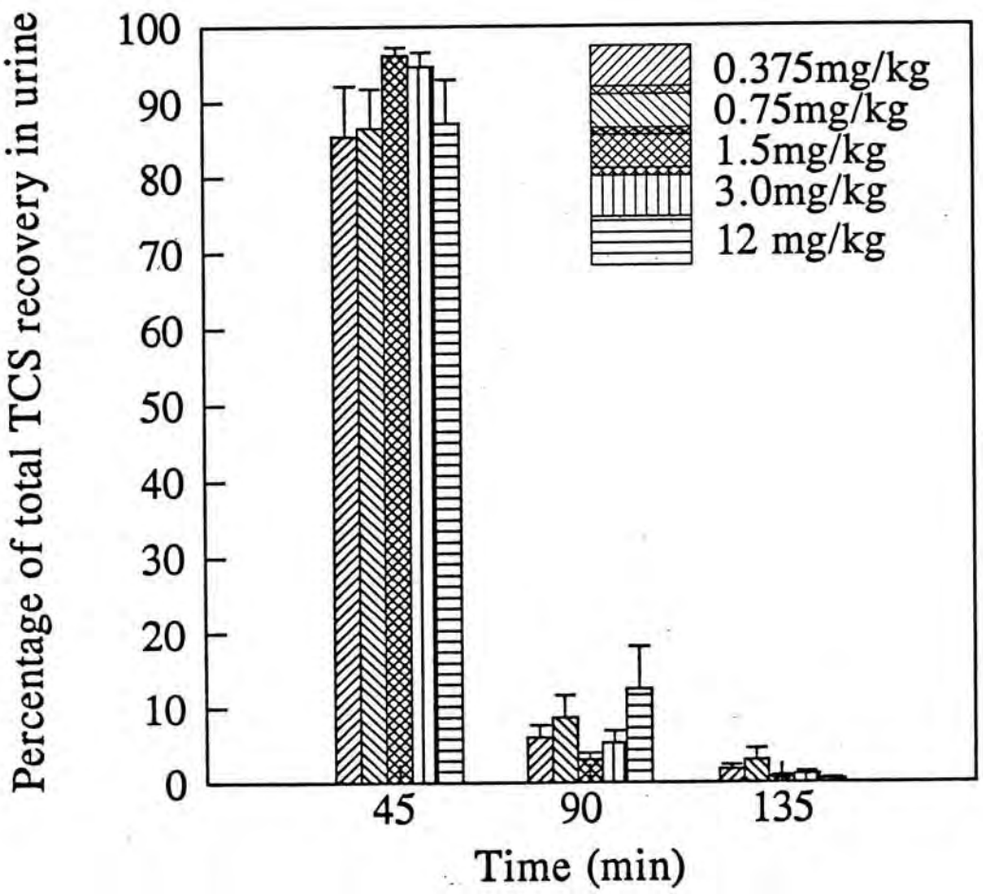


Fig. 6.3 Relative percentage of total TCS recovered in urine at 45 min interval. Values are expressed as means ± SEM.

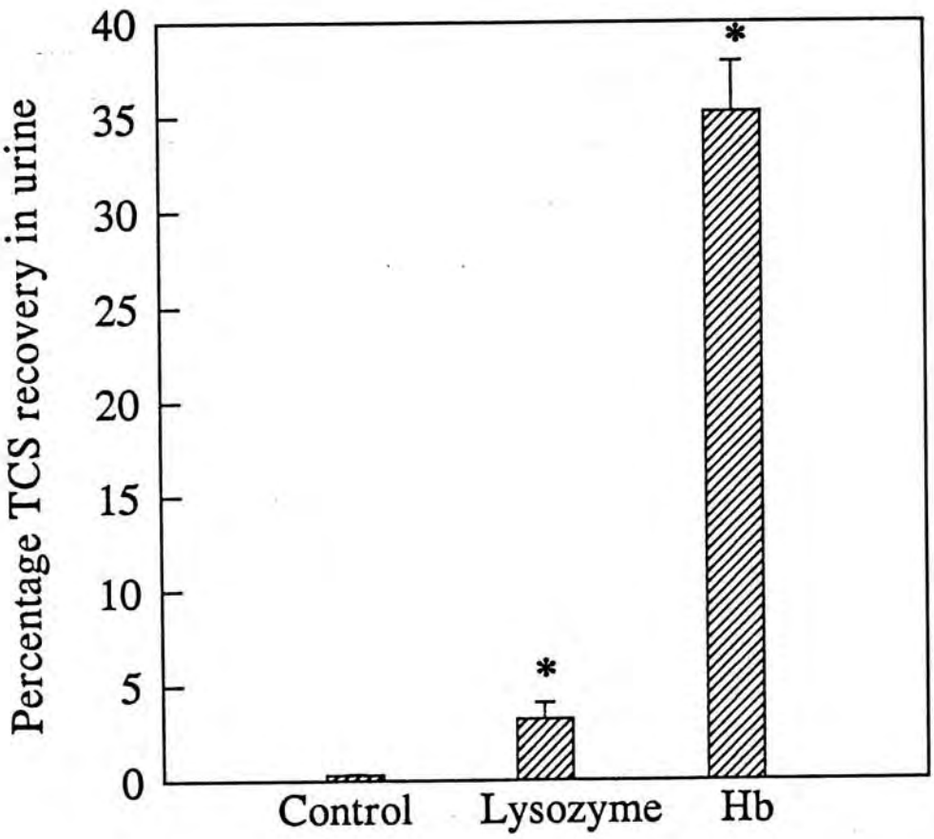


Fig. 6.4 Effect of infusing 77.5 μM lysozyme (N=12) or haemoglobin (N=14) at a rate of 3.4 ml/hr on urine TCS recovery. Trichosanthin was injected at a dose of 0.375 mg/kg. Values are expressed as means $\pm$ SEM.

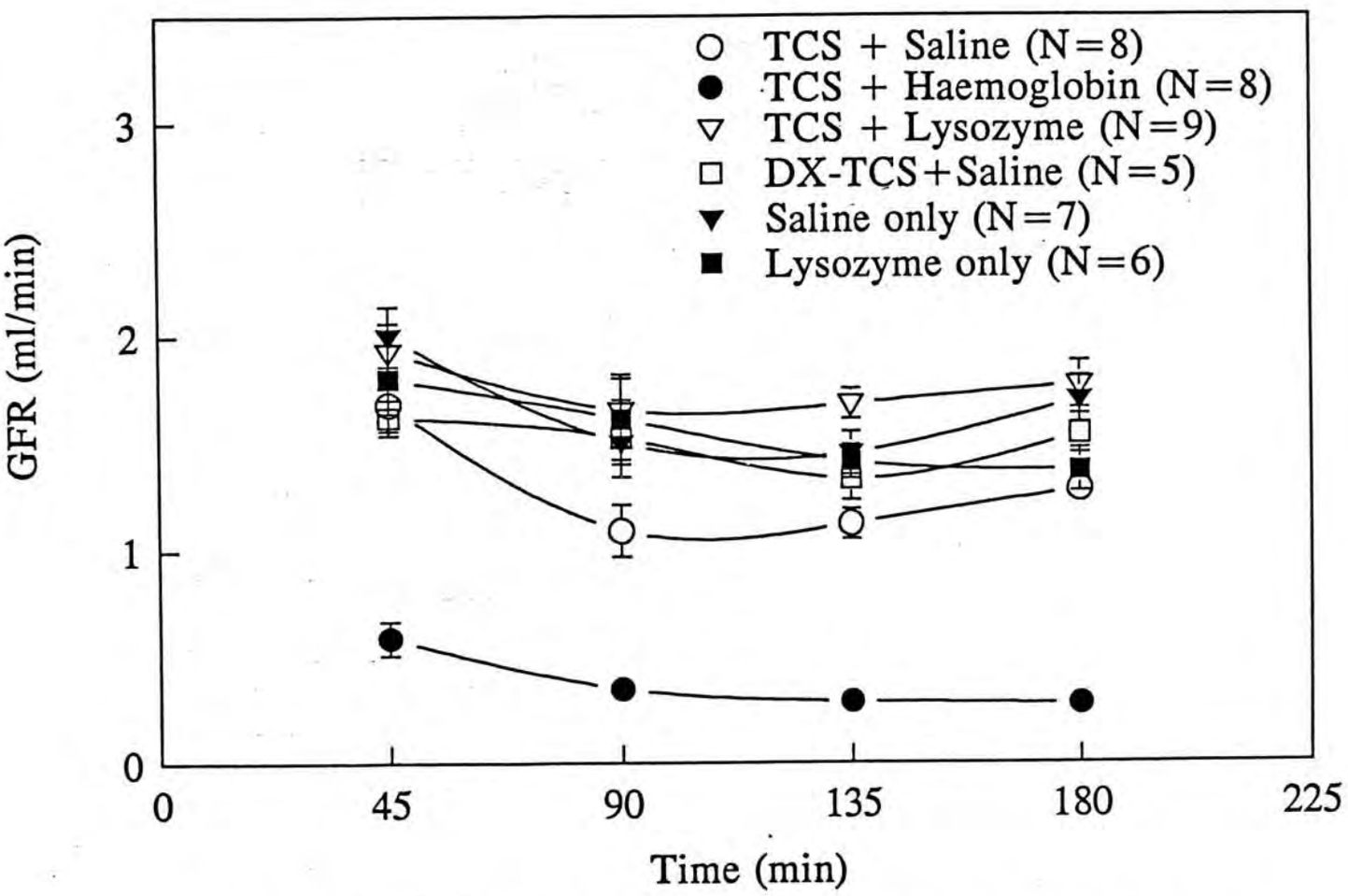


Fig. 6.5 Temporal profile of the inulin clearance in six groups of rat. Drug was injected at time zero. Infusion of saline, lysozyme, or haemoglobin started at 90 minutes before drug injection and continued throughout the experiment. Values are expressed as means ± SEM. Only (i) TCS injection and saline infusion (ii) TCS injection and haemoglobin infusion showed a significant ($p < 0.05$) decrease in GFR when compared with the control (saline only).

Discussion

The present experimental data support that the filtered TCS was reabsorbed by the kidney. The first line of evidence was that the recovery of TCS in urine could be increased by using higher injection dose. The percentage recovery also followed a dose dependent manner. If there was no reabsorption of TCS in the kidney, the amount of TCS filtered would equal to the amount excreted and hence the amount recovered in urine. The amount of TCS filtered depend on both the glomerular filtration rate and the plasma TCS concentration which equals to the total amount of drug injected divided by the distribution volume. Therefore, the percentage of TCS recovered in a certain period of time would equal to the amount of TCS filtered divided by the total amount of TCS injected which subsequently result in a mathematical relationship independent of the dosage of TCS. As a consequent, the percentage of TCS recovered should remain the same regardless of different injection doses if there is no tubular reabsorption. On the other hand, if small injection dose was used, the kidney was able to reabsorb most of the filtered TCS resulting in low urine recovery. On the contrary, the injection of large amount of TCS exceeded the renal reabsorptive capacity of TCS so that a lot of TCS was spilt into the urine. For similar reasoning, a major part of the urine TCS was recovered in the first 45 min after injection for all of the injected doses. It was because in this period, the plasma TCS concentration and hence the filtered load was highest. This exceed the capacity

of the kidney to reabsorb TCS and therefore relatively large amount of TCS was recovered from the urine. Afterwards, the plasma concentration decreased rapidly and the kidney was capable to reabsorb most of the TCS. Indeed, the proximal tubular cells are capable of reabsorbing up to 0.2 mg/kg body weight of small molecular weight proteins in 1 hour (90). This quantity is in the same order of magnitude as the amount of TCS administered.

Concerning the mechanism of tubular TCS reabsorption, it is logical to assume that TCS utilizes the common reabsorption process with other small molecular weight proteins. The body is unlikely to have a specific pathway for this exogenous protein which do not appear in the renal tubules in normal circumstances. Kidney is an important organ for the catabolism of most small molecular weight protein (e.g. lysozyme) (75,74,85) and also larger proteins such as albumin and haemoglobin (91). Proteins are reabsorbed by segregation into endocytotic vesicles at the apical border of tubular cells. The vesicles then migrate to the interior of the cells and fuse with lysosomes in which they are digested. The first step in endocytosis in the proximal tubule involves the binding of the positively-charged moiety of the protein to the negatively charged luminal membrane. Thus, this process can be inhibited by cationic but not anionic or neutral amino acids (75). Also, infusion of one protein can increase the excretion of another due to the sharing characteristic of the endocytotic process (86,87).

The competitive nature of TCS reabsorption can be demonstrated. Both the infusion of lysozyme and haemoglobin caused a significant increase in urinary TCS excretion. This enhancement effect is likely due to competition between the infused protein and TCS for the reabsorption process. This also implies that both lysozyme, haemoglobin and TCS shares the common endocytotic mechanism which is rather non-specific to the physico-chemical features of the protein.

From the inulin clearance data, it seemed that both the reabsorption of TCS and haemoglobin into renal tubular cells caused the gross malfunction of the kidney as manifested by the reduction in GFR which is an index of renal damage. Hence, they were nephrotoxic when presented in the tubular cells. In fact, it has been shown previously that presence of haemoglobin in renal tubules would induce kidney damage (88). However, this nephrotoxic effect could be avoided if we could prevent the reabsorption of TCS. Therefore, when DX-TCS was injected instead of TCS, GFR was the same as control because the conjugate was not able to pass through the glomerulus and reabsorbed. Simultaneous infusion of lysozyme also caused the GFR remained within the normal range probably by reducing its tubular reabsorption. Although lysozyme was also reabsorbed into tubular cells, it did not generate nephrotoxic effect. It seems that the nephrotoxicity was related to the nature of the reabsorbed molecule that is present inside the tubular cells rather than the reabsorption process. Therefore, the damaging effect of TCS may be attributed to its

ribosome inactivating property which takes place inside the tubular cells.

The precise relationship between tubular damage and the depression in glomerular filtration rate in this case was not known. However, it may attribute to the activation of the tubuloglomerular feedback mechanism of the kidney. The tubuloglomerular feedback is a regulatory mechanism which tends to lower the glomerular filtration rate whenever the solute delivery or solute concentration at the macula densa is raised (101, 102). After reabsorption of TCS and ribosome inactivation had taken place, it might impair the sodium reabsorption of the tubular cells. It would then result in an increase in macula densa flow and chloride delivery. Therefore, in order to prevent excessive salt and water losses, the kidney will initiate a response that involves afferent arteriolar constriction and decreased capillary permeability by contraction of mesangium. Consequently, a depression of glomerular filtration rate follows (103, 104). The damaging effect of TCS was very rapid and was most prominent at 90 minute after drug injection as reflected by the lowest glomerular filtration rate. This time interval was more or less similar to the time required for the physiologic effects of the steriod hormine aldosterone in which its action also includes cellular entry of the hormone and ultimately increasing the synthesis of new channel proteins that responsible for sodium reabsorption (107).

Chapter 7: Biological activities of Dextran-Trichosanthin

The previous chapters described the coupling of dextran to TCS and the pharmacokinetic properties of the conjugate. The next critical step thus involves the characterization of the biological activity of the conjugate. It is very important to know whether the DX-TCS conjugates still retain biological activities of the parent compound. With this regard, different bioassays (both *in vivo* and *in vitro*) were performed to evaluate whether coupling of TCS to dextran would alter its original biological behaviour.

Trichosanthin has wide spectrum of biological and pharmacological activities. An important one is the induction of mid term abortion (3,30,31). Hence, the *in vivo* bioassay selected was mid term abortifacient activity on pregnant mice. Other methods of evaluation includes *in vitro* bioassays in tissue culture. Trichosanthin is known to suppress the mitogenic effect on T and B lymphocytes (53,54), this immunosuppressive effect was compared between the native compound and the conjugate. Moreover, the anti-tumour activity of the conjugates was also evaluated using two different tumour cell lines (PU5-1.8 and H35).

Method

The general procedures of the different bioassays were described in methodology (Chapter 2). Briefly, the evaluation of mid term abortifacient activity was done by injecting different concentrations of TCS (0.01-0.20 mg/25g) and DX-TCS (0.01-0.40 mg/25g) intraperitoneally into mice on day 12 of pregnancy (PD 12). Mice were then autopsied on day 14 (PD 14) of pregnancy and the abortifacient effect was examined. Control mice were injected either with normal saline solution or dextran solution. For the *in vitro* bioassays, the target cells (T and B lymphocytes, tumour cells) were treated with various concentrations of TCS or DX-TCS and cultured for 48-hr period. The degree of cell proliferation were then measured by determining the rate of tritiated thymidine incorporated into DNA. Results were expressed as % inhibition with reference to the control using the following formulae:

$$\% \text{ inhibition} = 1 - \frac{^{3}\text{H-TdR incorporation in the presence of drug}}{^{3}\text{H-TdR incorporation in the absence of drug (control)}} \times 100\%$$

In order to clarify that the effect of TCS or DX-TCS is inhibitory on proliferation rather than cytotoxicity, in a separate experiment, normal target cells were cultured with a high concentration (50μg/ml for mouse lymphocytes and 100μg/ml for tumour cells) of drugs for 2 days. The cell viability after incubation was assessed by trypan blue dye exclusion method (76).

Statistical analysis

One way analysis of variance followed by Scheffe multiple comparison test was used to examine the cytotoxic effect of various drugs on lymphocytes and tumour cells.

Results

1. *in vivo* bioassay - Midterm abortifacient activity

The midterm abortifacient activity of TCS and its conjugates on pregnant mice was done and the results were summarized in Table 7.1. Mice were considered aborted when the no. of dead fetuses exceeded 50% of the total implantation sites. Results showed that TCS has potent midterm abortifacient activity. A single intraperitoneal (i.p.) TCS injection of as little as 0.01 mg/25 g body weight (B.W.) elicited 67% midterm abortion. DX-TCS, like its parent compound, could also induce midterm abortion. The potency of the conjugates in inducing midterm abortion was decreased. No abortifacient effect was detected at the dose of 0.05 mg/25 g B.W. DX-TCS while TCS injection of this dose could induce 100% midterm abortion. However, 100% midterm abortion was attained only when the injection dose of DX-TCS was increased to 0.40 mg/25 g B.W. For the control mice, injection of either normal saline solution or dextran solution showed no abortifacient effect. The toxicity

of the conjugates as judged from the mortality rate of the treated mice was also found to be decreased. A single dose of 0.10 mg/25 g B.W. TCS was toxic and about 33% of the treated mice was dead. In contrast, no death occurred in the treated mice received DX-TCS injection even when four times as much DX-TCS was used. Figure 7.2 is a photograph showing the gross morphology of the uterine implantation sites of a normal and a aborted mice while Fig. 7.3 shows a more closer examination of the normal and dead fetuses.

2. *in vitro bioassay*

(A) Mitogen induced lymphocyte transformation

Figure 7.4 - 7.6 showed the effect of TCS or DX-TCS on the % inhibition of tritiated thymidine uptake at various drug concentrations. The mitogenic responses of lymphocytes induced by Con A, PHA and LPS were inhibited by TCS as shown by the decreased incorporation of thymidine with increasing drug concentration. The mitogenic response was greatly suppressed ($\geq$ 90%) at TCS concentration of 50 μg/ml and was clearly dose dependent. When comparing the three figures, TCS appeared to be more inhibitory to the PHA response according to their respective IC_{50} which refers to the concentration of drug that cause 50% inhibition. Figure 7.4 shows that significant suppression was seen at a protein concentration of as low as 0.1 μg/ml. It took only 5 μg/ml of TCS to attain 50% inhibition which was less than that of Con A (10 μg/ml) and LPS (7 μg/ml).

induce significant inhibition. Fig. 7.11 showed the response of another cell line (rat hepatoma cells) to TCS and DX-TCS. From the graph, it was found that hepatoma cells were relatively resistant to the cytostatic effect of TCS with the approximate ID_{50} of 10 μg/ml. The ID_{50} of DX-TCS was 50 μg/ml. However, both the dextran and DX-TCS produced similar cytostatic effect on the cells.

(A) TCS

Dose (mg/25g)	No. of treated mice	No. of dead fetuses/ No. of implantation sites	% of aborted mice*	No. of dead mice
0.20	7	40/40 (100%)	100%	5
0.10	12	111/111 (100%)	100%	4
0.05	4	55/55 (100%)	100%	0
0.02	4	51/51 (100%)	100%	0
0.01	3	31/38 (38%)	67%	0

(B) Dx-TCS

Dose (mg/25g)	No. of treated mice	No. of dead fetuses/ No. of implantation sites	% of aborted mice*	No. of dead mice
0.40	7	102/102 (100%)	100%	0
0.20	10	129/143 (90%)	90%	0
0.10	11	115/168 (68%)	55%	0
0.05	6	7/86 (8%)	0%	0
0.02	5	13/68 (19%)	0%	0
0.01	3	9/43 (21%)	0%	0

(C) Control

Normal Saline/ Dextran Solution	No. of treated mice	No. of dead fetuses/ No. of implantation sites	% of aborted mice*	No. of dead mice
Normal Saline	18	14/261 (5%)	0%	0
Dx(0.2mg/25g)	9	4/126 (3%)	0%	0
Dx(0.4mg/25g)	2	3/16 (19%)	0%	0

*Mice was considered aborted when the no. of dead fetuses exceeded 50% of the total implantation sites.

Table 7.1 Midterm abortifacient activity of TCS and DX-TCS on pregnant mice.

Fig. 7.3 A photograph showing the gross morphology of the uterine implantation sites of a normal (left) and a aborted mice (right).

Fig. 7.4 A photograph showing the normal fetuses (1) and dead fetuses (2) dissected out from the uterine implantation sites.

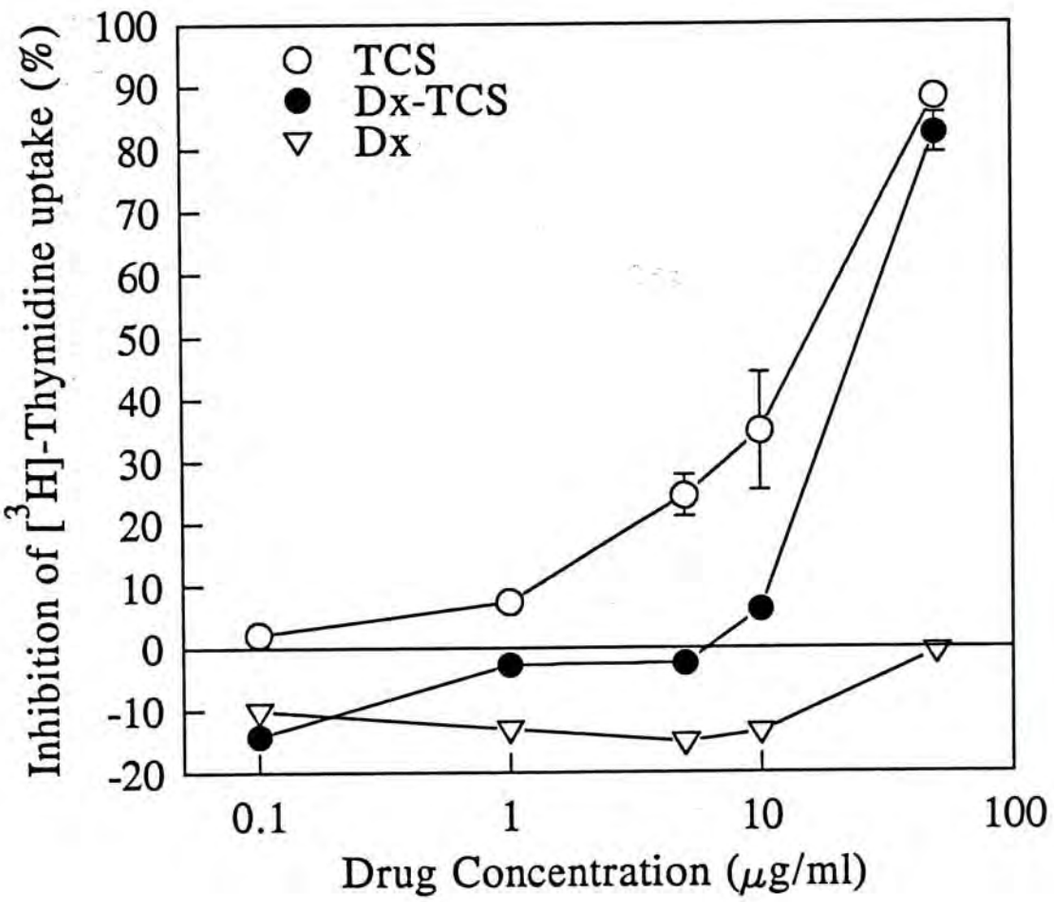


Fig. 7.4 Effect of TCS, DX-TCS and dextran on Con A-induced lymphocyte transformation. Concentrations of TCS and dextran were adjusted for individual components. Values are expressed as means ± SEM (N=4).

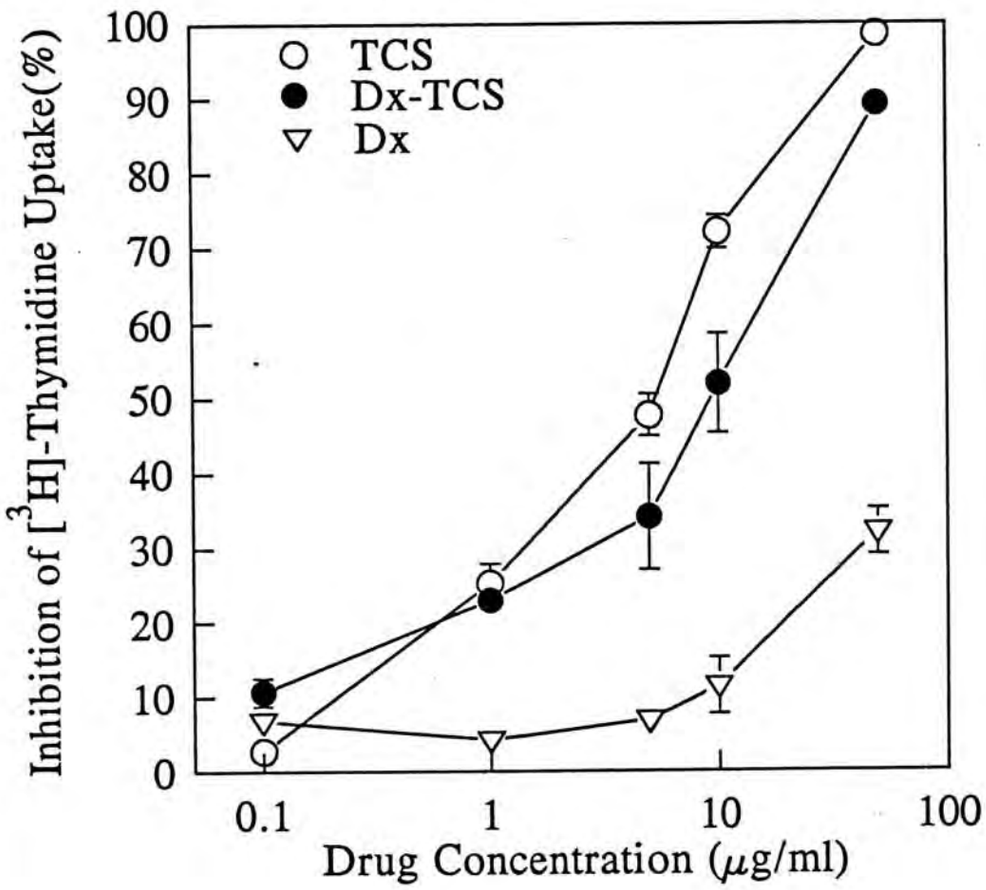


Fig. 7.5 Effect of TCS, DX-TCS and dextran on PHA A-induced lymphocyte transformation. Concentrations of TCS and dextran were adjusted for individual components. Values are expressed as means ± SEM (N=4).

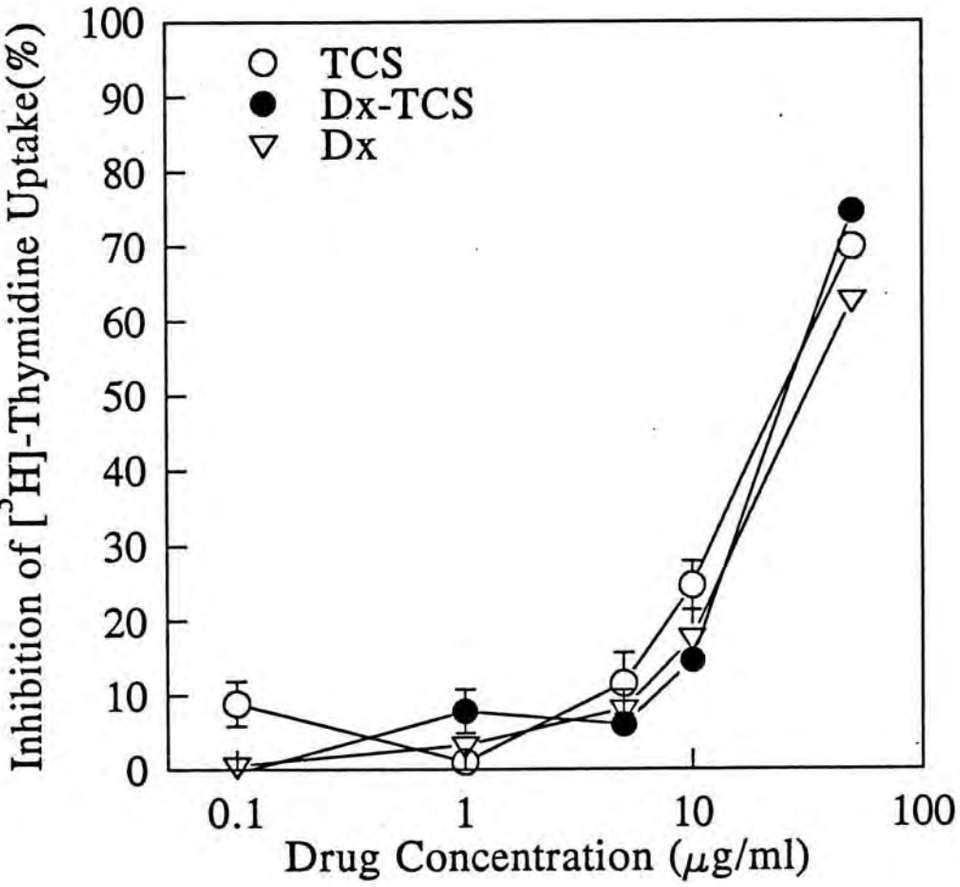


Fig. 7.6 Effect of TCS, DX-TCS and dextran on LPS-induced lymphocyte transformation. Concentrations of TCS and dextran were adjusted for individual components. Values are expressed as means ± SEM (N=4).

Drug	Conc.(μg/ml)	% of cells surviving after 48 hrs
Control	0 μg/ml	92.72 $\pm$ 0.74
TCS	50 μg/ml	90.66 $\pm$ 1.53
DX-TCS	50 μg/ml	86.24 $\pm$ 0.65
Dextran	50 μg/ml	83.44 $\pm$ 0.90

Table 7.7 The effect of TCS, DX-TCS and dextran on the % viability of lymphoyctes. No 2 groups are significantly different at p=0.05 using oneway analysis of variance followed by Scheffe Multiple Comparison test (N=6).

Drug	Conc.(μg/ml)	% of cells surviving after 48 hrs
Control	0 μg/ml	92.51 $\pm$ 2.80
TCS	100 μg/ml	86.67 $\pm$ 5.60
DX-TCS	100 μg/ml	98.18 $\pm$ 1.82
Dextran	100 μg/ml	97.33 $\pm$ 2.67

Table 7.8 The effect of TCS, DX-TCS and dextran on the % viability of PU5 cells. No 2 groups are significantly different at p=0.05 using oneway analysis of variance followed by Scheffe Multiple Comparison test (N=6).

Drug	Conc.(μg/ml)	% of cells surviving after 48 hrs
Control	0 μg/ml	96.18 $\pm$ 1.98
TCS	100 μg/ml	93.88 $\pm$ 2.39
DX-TCS	100 μg/ml	95.68 $\pm$ 1.00
Dextran	100 μg/ml	93.55 $\pm$ 3.29

Table 7.9 The effect of TCS, DX-TCS and dextran on the % viability of hepatoma cells. No 2 groups are significantly different at p=0.05 using oneway analysis of variance followed by Scheffe Multiple Comparison test (N=6).

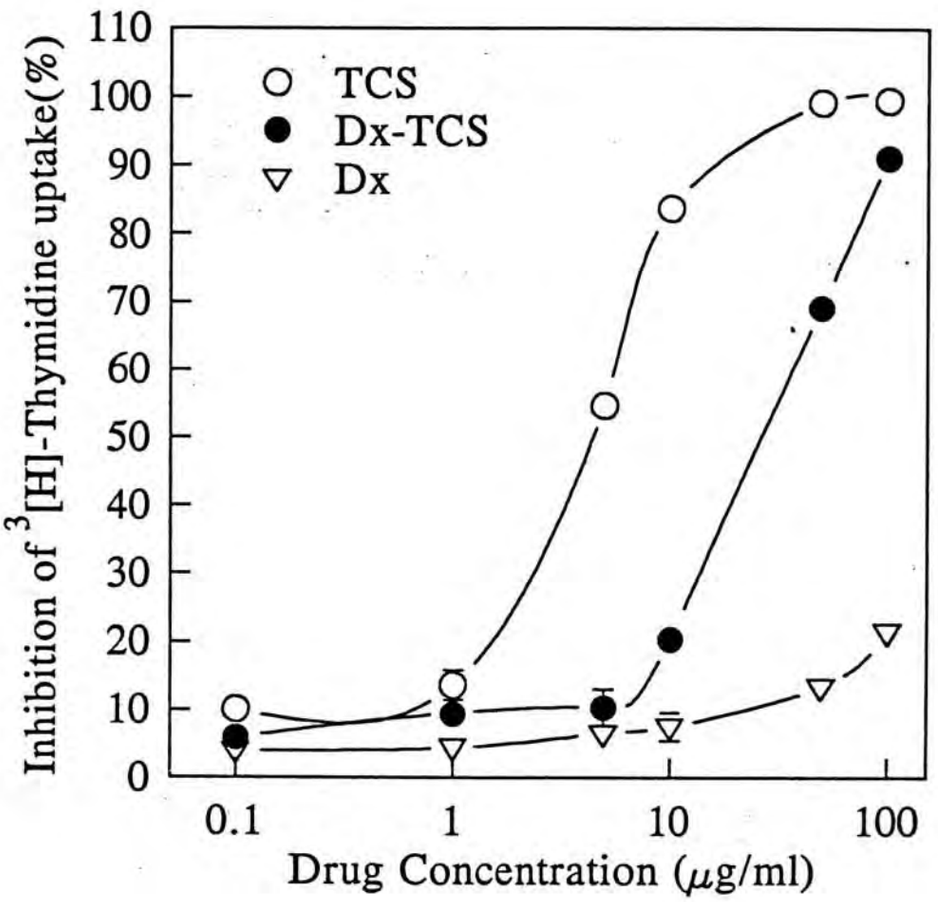


Fig. 7.10 The cytostatic effect of TCS, DX-TCS and dextran on the growth of PU5 cells after incubating with the drugs for 48 hr. Values are expressed as means ± SEM (N=4).

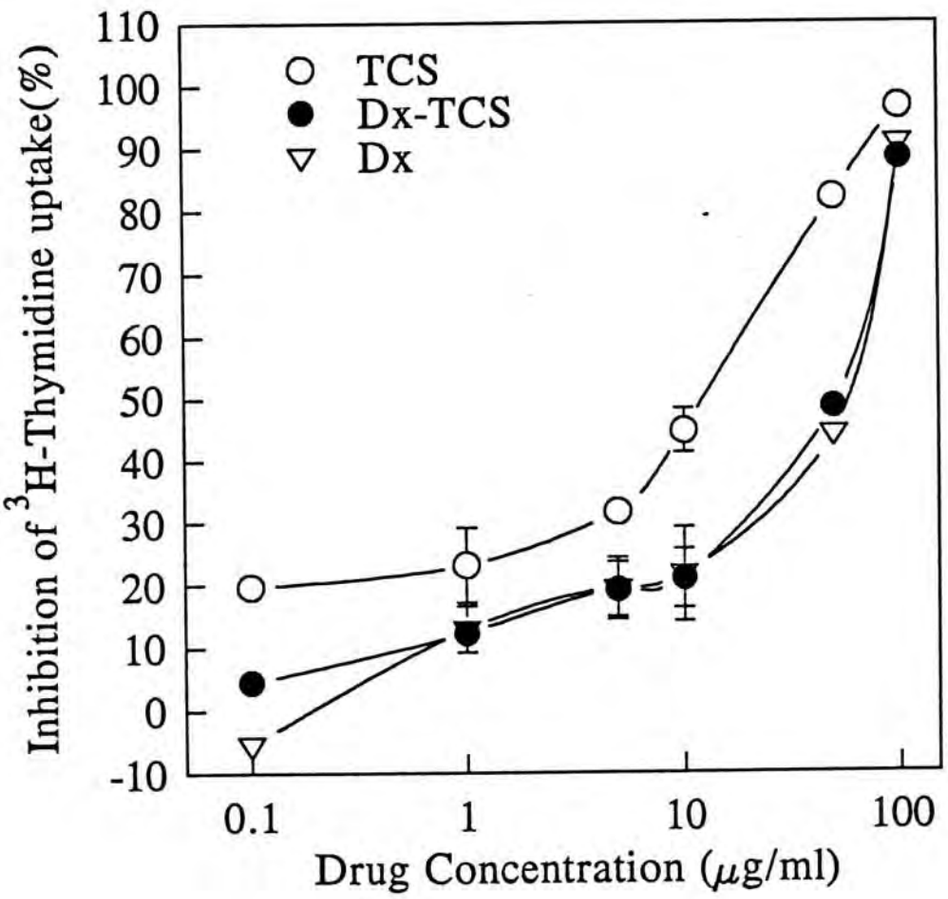


Fig. 7.11 The cytostatic effect of TCS, DX-TCS and dextran on the growth of hepatoma cells after incubating with the drugs for 48 hr. Values are expressed as means ± SEM (N=4).

Discussion

The biological activities of DX-TCS conjugate as determined by both *in vivo* and *in vitro* bioassays were well preserved. However, there was a general decrease in potency when compared to the native compound. For example, in midterm abortion, it took twenty times as much DX-TCS than TCS to induce 100% abortion. The ability of DX-TCS to inhibit Con A and PHA induced lymphoproliferative response was decreased. DX-TCS was six times less potent than its native compound in inhibiting the growth of PU5 cells as estimated from their respective ID_{50}.

The detail mechanism of this decrease in potency was still unknown at this stage. However, the most logical speculation is that the stearic rigidity acquired by the dextran conjugate is responsible for the decreased pharmacological activity. Loss of activity can also due to the blocking of active sites during the coupling reaction. Trichosanthin is a single chain Type I RIP which has to get into the cells to exert its inhibitory action on protein synthesis (18,19,20). Therefore, TCS is most sensitive to cells which are phagocytotic in nature. For those target cells (e.g. PU5 cells, syncytiotrophoblast in the placenta) which are phagocytotic in nature, DX-TCS conjugate is still able to enter these type of cells where it exerts its action. As a consequence, the biological activities can be preserved although with decreased potency.

In the study of anti-tumour activity, PU5 cells were more sensitive than hepatoma cells to the cytostatic action of the drugs as evaluated from their respective ID_{50}. This is probably due to the fact that PU5 is a macrophage-like cell line and was expected to possess phagocytotic ability. This would facilitate the internalization of the drugs into the cell which was thought to be a prerequisite for the inhibitory action on tumour growth. In fact, TCS has been used therapeutically for the treatment of certain types of trophoblastic tumours (37).

Similarly, the ID_{50} of DX-TCS in inhibiting PU5 and hepatoma cells were 30 and 50 μg/ml respectively indicating that the conjugate was less effective against hepatoma cells. Since dextran also produced similar inhibitory effect on hepatoma cells, the anti-tumour activity could either due to the TCS component, dextran component or both. Nonetheless, the protein component of DX-TCS was less effective in inhibiting hepatoma cells than PU5 cells because of the increased ID_{50} and some of its inhibitory effect might attribute to the dextran component. Therefore, coupling of TCS to dextran seems to further reduce its cellular entry into the non-phagocytotic cells. This is probably due to the inability of the larger complex to enter this type of cells which are non-phagocytotic in nature. On the contrary, coupling of TCS to dextran did not affect the phagocytosis of the PU5 cells although the molecular size was expanded.

Apart from the potency of the conjugate, its toxicity was also decreased. In the midterm abortifacient activity, a dose of as little as 0.1 mg/25g B.W. induced 33% mortality whereas four times as much DX-TCS (0.4 mg/25g) did not cause any death. With regard to the effect of coupling on cellular entry of TCS, the reduction in toxicity may due to the inability of the conjugate to enter other normal cells. Therefore, coupling may probably channel the conjugate to the phagocytotic target cells while reducing the non-specific damaging on other cells. This reduction in toxicity is indeed valuable in improving the therapeutic usage of the drug.

In conclusion, after coupling of TCS to dextran, the product is still biologically active. It retained most of the biological activities of the parent compound evaluated by different bioassays, both *in vivo* and *in vitro*. Although the effectiveness was decreased, its toxicity was also decreased suggesting an improved therapeutic usage of the drug after modification.

Chapter 8: Immunological activities of DX-TCS

Previous chapters described that DX-TCS has a longer biological half-life than its native compound with reduced biological activities and toxicity. Base on the clinical experience in China, extensive clinical usage of TCS is limited by its allergic side effect (e.g immediate hypersensitivity; 93,94). For this reason, skin test has to be done in these patients prior to the administration of this drug. In this study, an attempt is made to examine whether coupling TCS to dextran can reduce its immunogenicity through the blocking of epitope(s) on the molecule.

After modification, the immunological activities of DX-TCS was examined by two different ways. Firstly, the binding of DX-TCS to TCS specific antibody is examined by radioimmunoassay. This is followed by a binding study using DX-TCS with the dextran moiety digested by the enzyme dextranase to see whether the dextran component of the DX-TCS molecule is responsible for the changes of binding affinity. Secondly, TCS, DX-TCS or dextran with/or without adjuvant were used to immunize the mice to examine the potency of these antigens in inducing IgG and IgE production by using an antibody capture approach with enzyme linked immunosorbent assay (ELISA). These two antibodies were chosen because they are important in determining the antigenicity of a compound. Immunoglobulin G is the major antibody of secondary immune response which is characterized by a sustained high titre of

antibodies. On the other hand, IgE takes an important role in mediating immediate hypersensitivity including anaphylaxis which may result in death of the animal.

Method

The binding characteristics of DX-TCS to TCS antibodies was firstly examined using a radioimmunoassay procedure in which DX-TCS standards instead of TCS were used. During the incubation, iodinated TCS competes with DX-TCS to bind to the TCS specific antibodies. This is followed by the examination of the binding characteristics of DX-TCS after the dextran moiety being digested by dextranase. (Dextranase is the enzyme that would hydrolyse the α-1→6 glycosidic linkage of dextran.) This was done by incubating DX-TCS with dextranase for 20 hr at 36°C. In the control experiment, dextran, dextranase or both were added to the radioimmunoassay standard tubes to see whether these substances would interfere the antibody-antigen reaction.

In the immunogenicity study of TCS, DX-TCS and dextran, mice (C57 BL/6N) were divided into 4 groups. The first 3 groups were immunized with (i) 10 μg TCS (ii) an amount of DX-TCS equivalent to 10 μg TCS (iii) dextran equivalent to the amount present in DX-TCS. The fourth group served as control with no immunization. Each antigen with complete Freund's adjuvant (1:1 v/v) was given to

a group of mice followed by 2 booster injections of the same antigen (with Freund's incomplete adjuvant) at 3 and 2 weeks afterwards. After the immunization procedure, TCS specific antibodies were detected by ELISA using antibodies capture approach. Briefly, TCS, DX-TCS or dextran was coated onto the microtitre plates. This was followed by blocking of excess binding sites and then incubated with diluted mouse sera. The IgG and IgE present in the mouse sera was detected by sheep anti-mouse IgG-HRP or sheep anti-mouse IgE and Donkey anti-sheep IgG-HRP respectively. Eventually, the bound enzyme (HRP) is visualized by the addition of chromogen (OPD) which is a colourless substrate that acts on the enzyme to produce a coloured end-product. The amount of antibody was then measured by assessing the amount of end-product through optical density scanning of the microtitre plate. The data obtained are quantified by comparing the respective titre of the mouse sera. For IgG quantitation, titre is defined as the greatest dilution giving an optical density of 0.8 unit. For IgE quantitation, titre is defined as the greatest dilution giving an optical density of 0.2 unit. The intra-assay coefficient of variance was determined by measuring the same serum sample twelve times in one assay. The inter-assay coefficient of variance was determined by measuring the same serum sample in triplicate in one run and repeated on 5 different assays.

Result

1. Binding activity of DX-TCS conjugate to TCS antibodies

Figure 8.1 shows the effect of dextran and/or dextranase on TCS competitive binding curve. The radioimmunoassay for TCS was not affected by the presence of dextran, dextranase or both. When compared the binding curve of DX-TCS to that of TCS, it shifted in a parallel manner to the right (Fig. 8.2) suggesting a decrease in affinity of DX-TCS to TCS antibodies. However, the binding affinity of DX-TCS was increased partially when the dextran moiety was digested by dextranase as shown by a back shift of the binding curve towards that of TCS.

2. Immunogenicity of TCS, DX-TCS and dextran

(a) With adjuvant

Before the assay was used to test the different mouse sera, its validity was checked by using hyperimmune mouse sera as positive control which has been characterized by radioimmunoassay. Result indicated that the TCS specific IgG and IgE could be detected by the TCS coated microtitre plate in this assay system and dilution curves were obtained as in Fig. 8.3.

Figure 8.4 - 8.6 shows the serial dilution curves of different mouse sera for IgG determination on TCS, DX-TCS and dextran coated ELISA plate respectively. The titres of different mouse sera performed in various ELISA plate coating were compared semi-quantitatively and summarized in Table 8.9a. Several features are evident from this table. First, mice immunized with TCS resulted in a titre of two-fold higher than those of mice immunized with DX-TCS on TCS coated microtitre plate. Second, when the sera were tested on DX-TCS coated microtitre plate, DX-TCS immunization resulted in two-fold more IgG bound onto the plate than that of TCS. Third, when the ability of the antigen in generating antibodies against itself was compared (i.e. TCS immunized sera on TCS coated plate compared with DX-TCS immunized sera on DX-TCS plate), DX-TCS immunization was found to produce approximately four-fold lower IgG than those of mice immunized with TCS. Fourth, the control mice and the mice immunized with dextran showed no immune response against TCS, DX-TCS or dextran. Finally, when mouse sera were tested on the dextran coated microtitre plate, only the mice received DX-TCS injection elicited trace amount of anti-dextran IgG.

Apart from the IgG response, the immunogenicity of TCS, DX-TCS and dextran in producing IgE was also performed and the respective serial dilution curves were shown in Figure 8.7 - 8.8. The comparisons between different combinations were summarized in Table 8.9b. On TCS coated microtitre plate, the sera of mice

immunized with TCS showed about eight-fold higher titre than did the sera of mice immunized with DX-TCS. Similar to that of IgG, there was two-fold difference in titre when the sera were tested on DX-TCS coated microtitre plate. When the ability of the antigen in generating antibodies against itself was compared (i.e. TCS immunized sera on TCS coated plate compared with DX-TCS immunized sera on DX-TCS plate), DX-TCS immunization was found to produce approximately four-fold lower IgG than those of mice immunized with TCS. Moreover, all the mice showed negative IgE response towards dextran.

The intra-assay coefficient of variance and the inter-assay coefficient of variance estimated for different assays were summarized in Table 8.10.

(b) Without adjuvant

Generally, the immune response was weaker as shown by a lower OD value when adjuvant was not used in immunizing the mice and the resulting pattern was also different. In the production of IgG, the serial dilution curve of TCS showed no significant difference with that of DX-TCS on either TCS or DX-TCS coated microtitre plate (Fig. 8.11 and 8.12). Similar to that with adjuvant, only DX-TCS injection produced trace amount of anti-dextran IgG (Fig. 8.13).

In the IgE response, the two groups were not significantly different from each other on the TCS coated microtitre plate (Fig. 8.14). On the contrary, DX-TCS immunization resulted in a four-fold higher IgE titre compared to TCS injection in DX-TCS coated microtitre plate (Fig. 8.15). The results for different combinations were simplified and summarized in Table 8.16. When the competence of different antigen in eliciting their specific antibodies was compared (i.e. TCS immunized sera on TCS coated plate compared with DX-TCS immunized sera on DX-TCS plate), DX-TCS immunization was found to produce approximately four-fold lower IgG and two-fold higher IgE than those of mice immunized with TCS. Moreover, all the mice failed to elicit anti-dextran IgE.

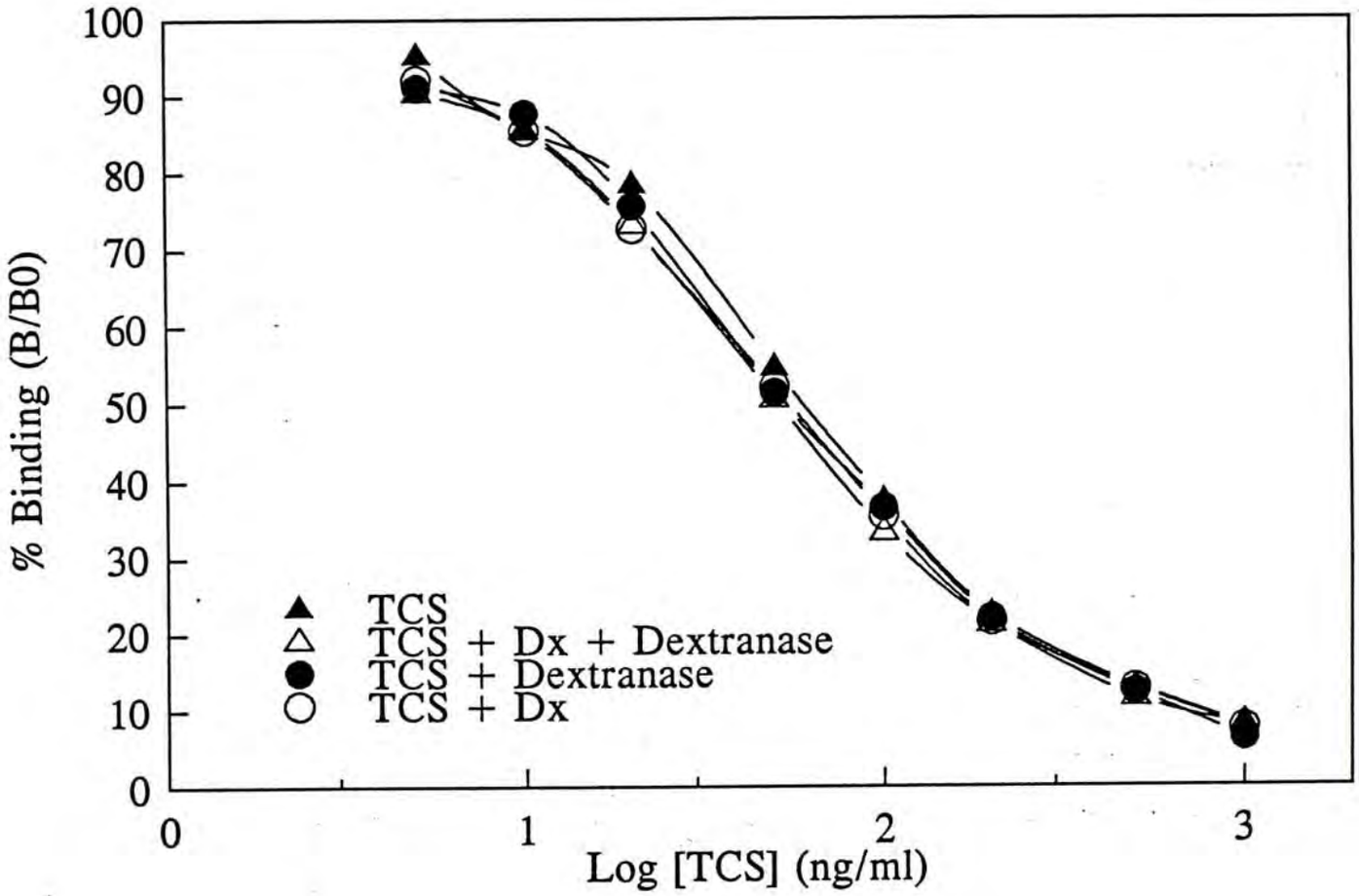


Fig. 8.1 The effect of dextran and/or dextranase on the TCS competitive binding curve.

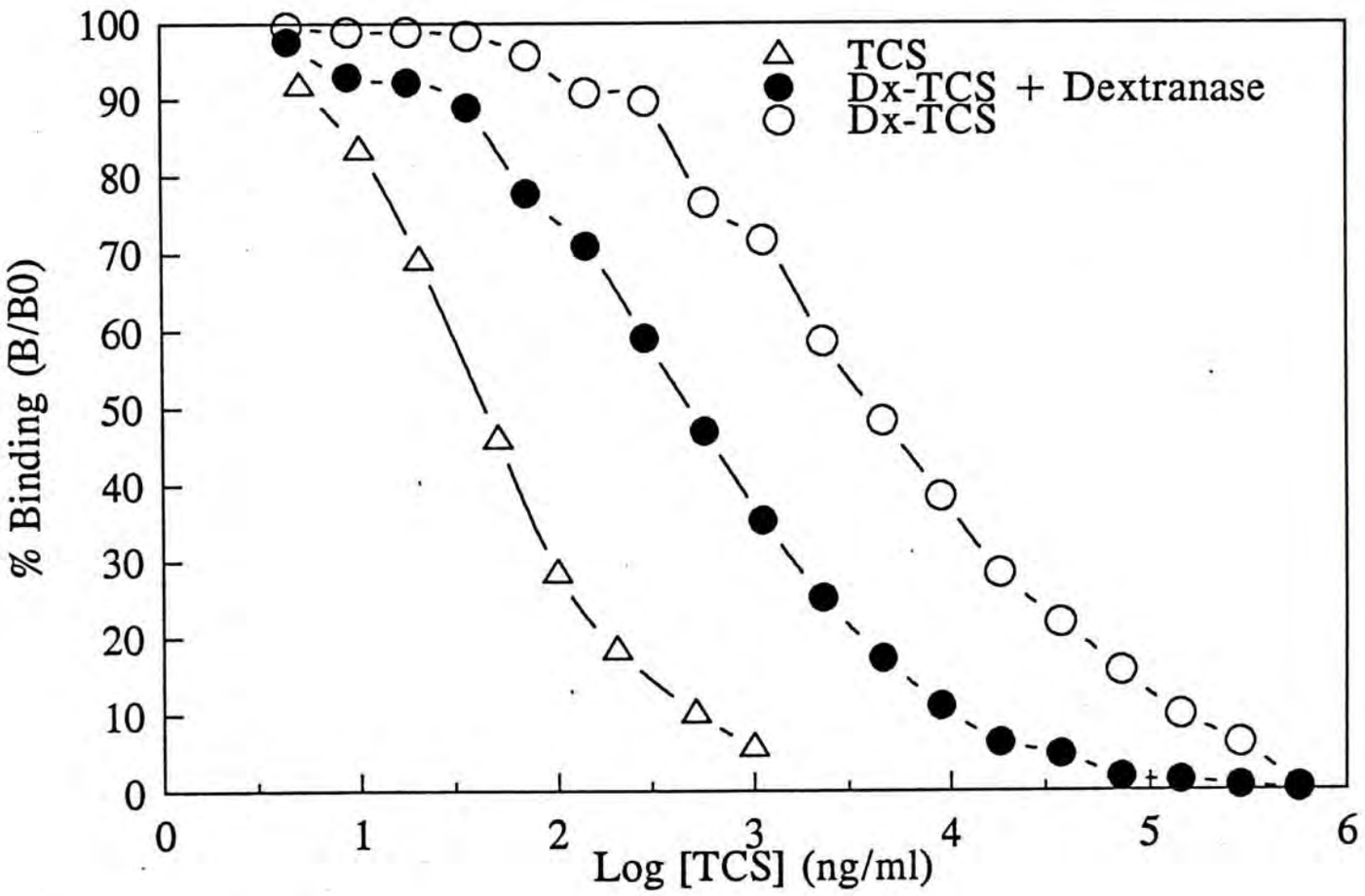


Fig. 8.2 The effect of dextranase on the DX-TCS competitive binding curve. Dextranase was incubated with DX-TCS for 20 hr.

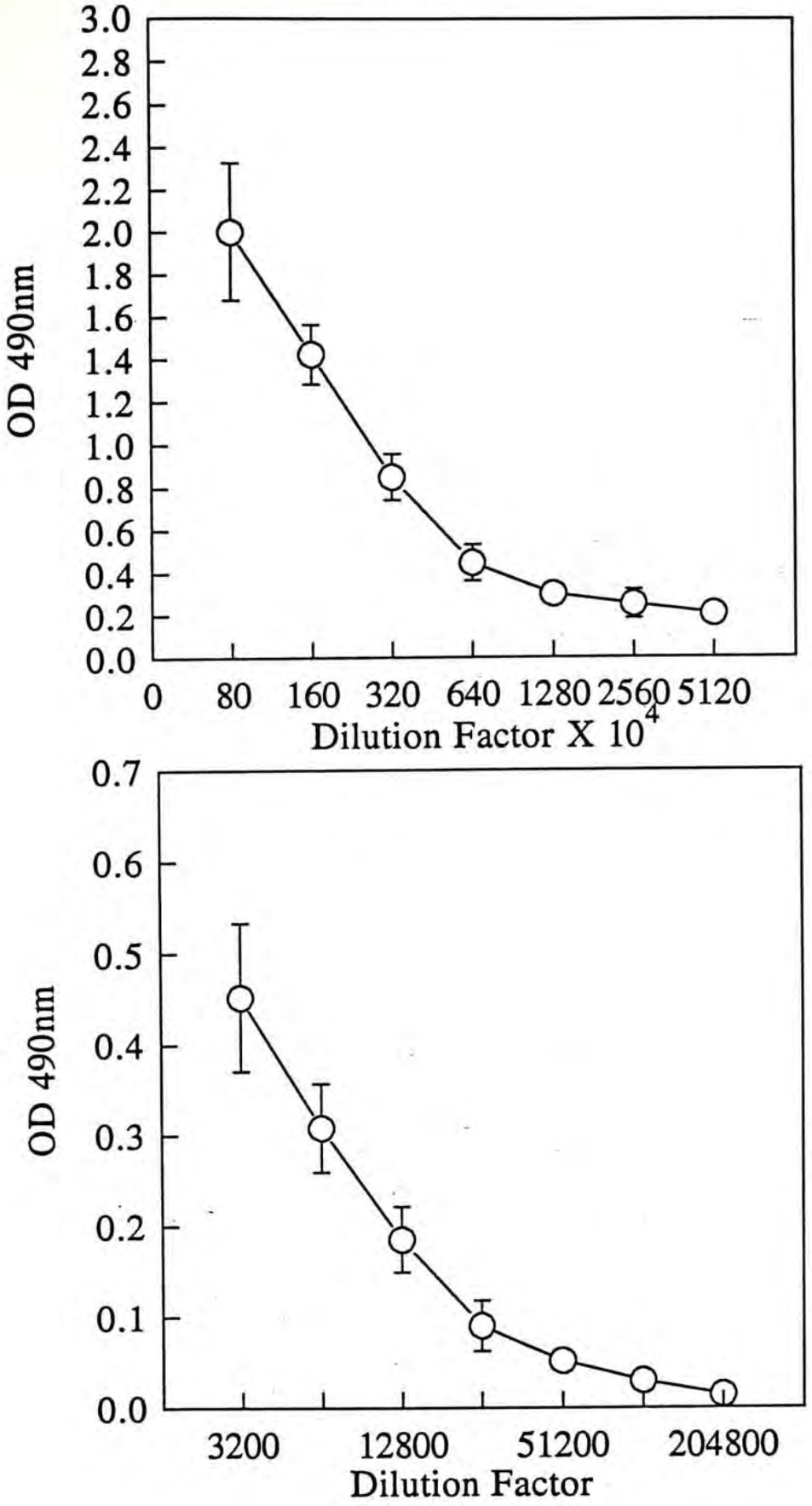


Fig. 8.3 Dilution curves of hyperimmune mouse sera. TCS specific IgG (upper) and IgE (lower) were detected on the TCS coated microtitre plate. Values are expressed as means ± SEM (N=5).

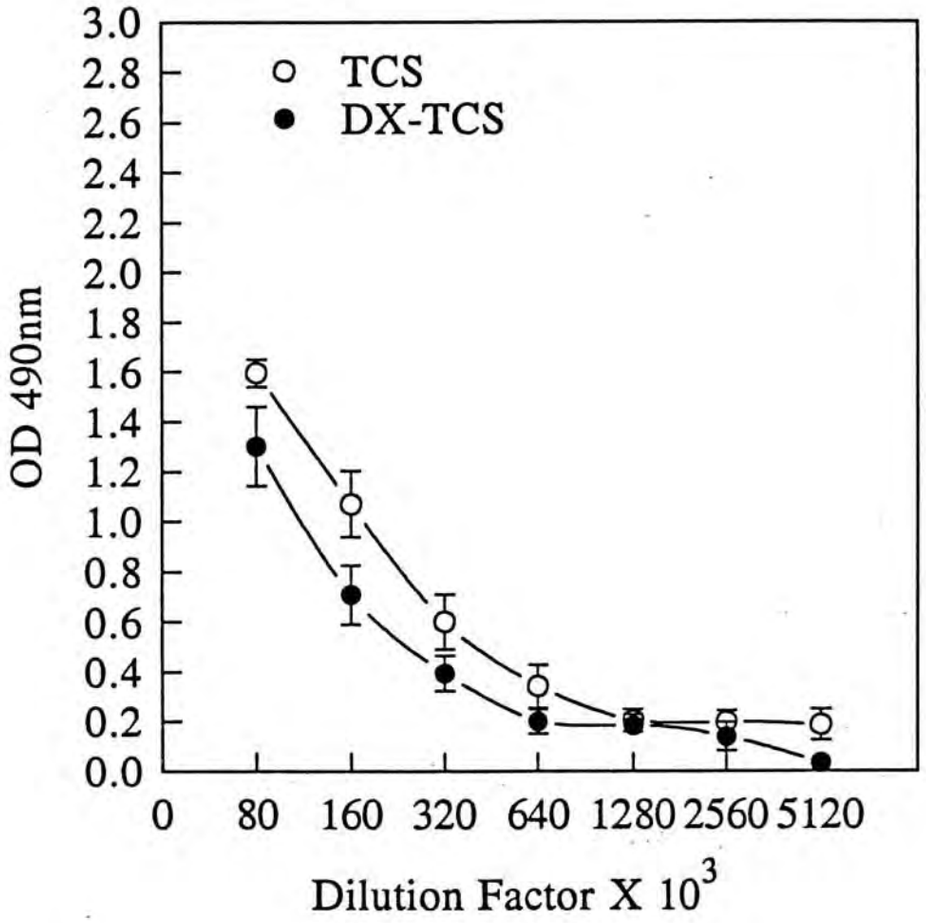


Fig. 8.4 Two-fold serial dilution curves of different mouse sera on TCS coated microtitre plate for IgG determination (with adjuvant). Values are expressed as means ± SEM (TCS, N=4; DX-TCS, N=5).

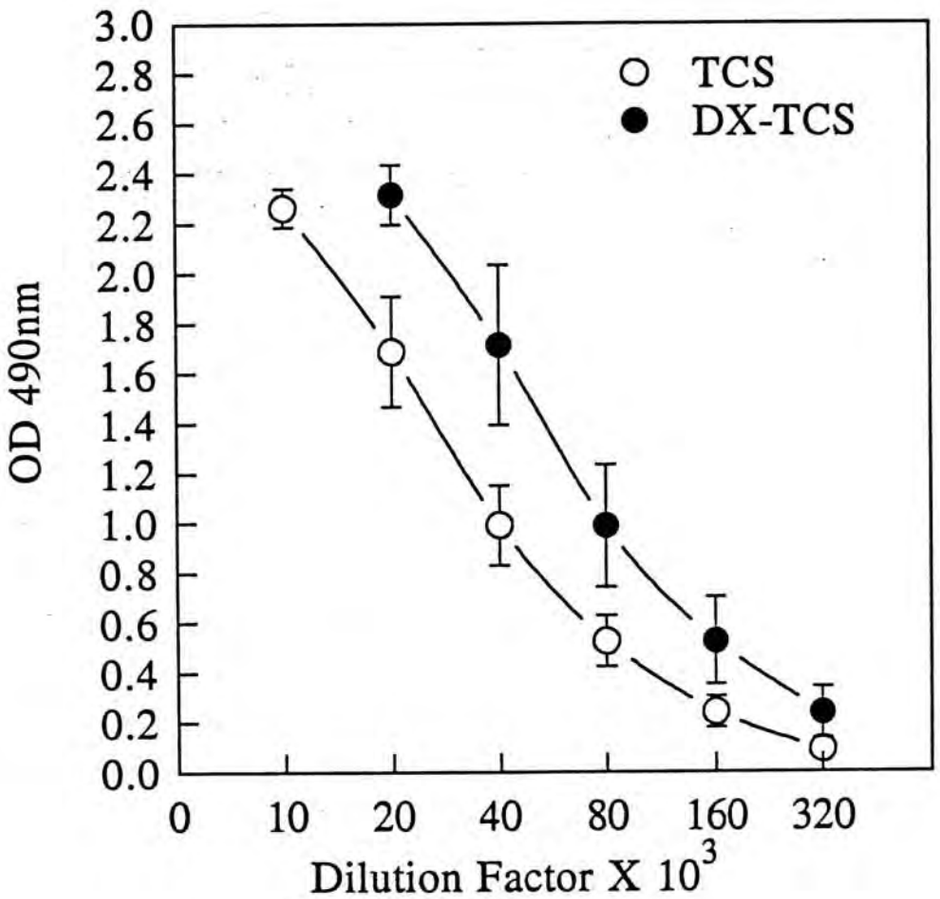


Fig. 8.5 Two-fold serial dilution curves of different mouse sera on DX-TCS coated microtitre plate for IgG determination (with adjuvant). Values are expressed as means ± SEM (TCS, N=4; DX-TCS, N=5).

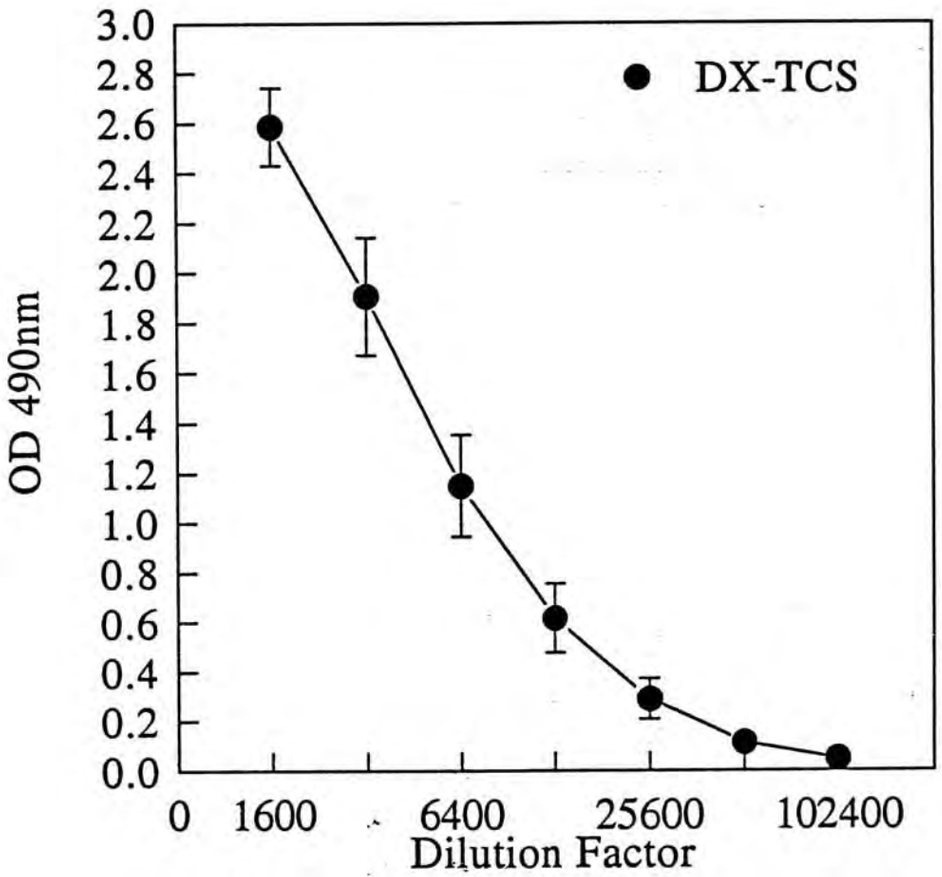


Fig. 8.6 Two-fold serial dilution curves of different mouse sera on dextran coated microtitre plate for IgG determination (with adjuvant). Values are expressed as means ± SEM (N=5).

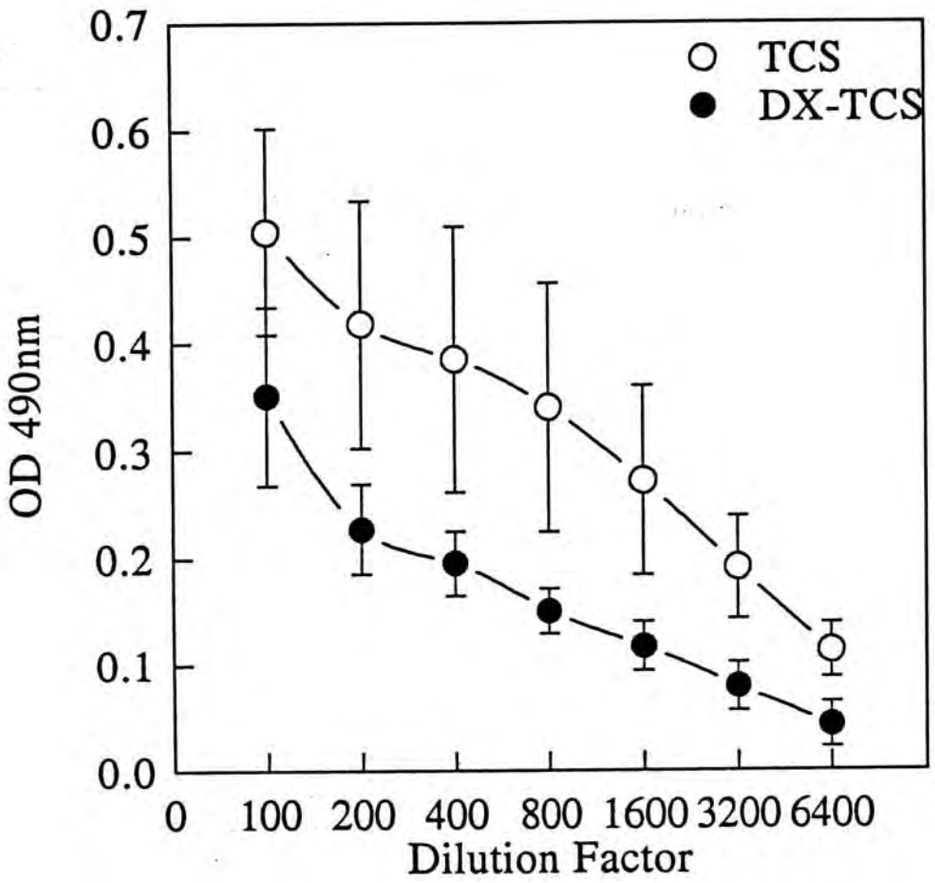


Fig. 8.7 Two-fold serial dilution curves of different mouse sera on TCS coated microtitre plate for IgE determination (with adjuvant). Values are expressed as means ± SEM (TCS, N=4; DX-TCS, N=5).

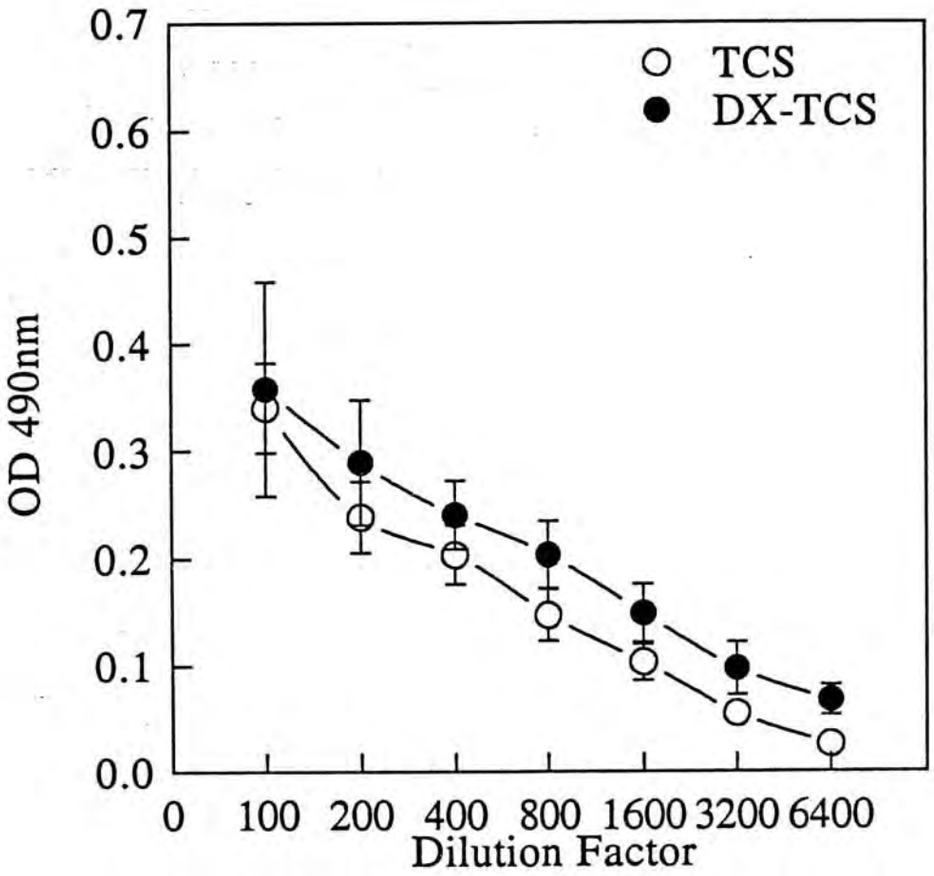


Fig. 8.8 Two-fold serial dilution curves of different mouse sera on DX-TCS coated microtitre plate for IgE determination (with adjuvant). Values are expressed as means ± SEM (TCS, N=4; DX-TCS, N=5).

(a) IgG

	COATING		
	TCS	DX-TCS	Dextran
(1) TCS	+ + + + + + + +	+	-
(2) DX-TCS	+ + + +	+ +	trace
(3) Dextran	_	_	_
(4) Control	_	_	_

(b) IgE

	COATING		
	TCS	DX-TCS	Dextran
(1) TCS	+ + + + + + + +	+	-
(2) DX-TCS	+	+ +	-
(3) Dextran	_	_	_
(4) Control	_	_	_

Table 8.9 A summary of relative quantity of IgG (upper) and IgE (lower) obtained from Fig. 8.4 - 8.8 in which different mouse sera (with adjuvant) were tested on various antigen coated microtitre plate.

(a) Intra-assay coefficient of variance

	Coating		
	TCS	DX-TCS	Dextran
IgG	3.82%	2.52%	3.66%
IgE	3.35%	2.90%	NIL

(b) Inter-assay coefficient of variance

	Coating		
	TCS	DX-TCS	Dextran
IgG	7.61%	3.66%	3.66%
IgE	4.15%	6.27%	NIL

Table 8.10 Intra-assay (a) and inter-assay (b) coefficient of variances in different enzyme linked immunosorbent assays.

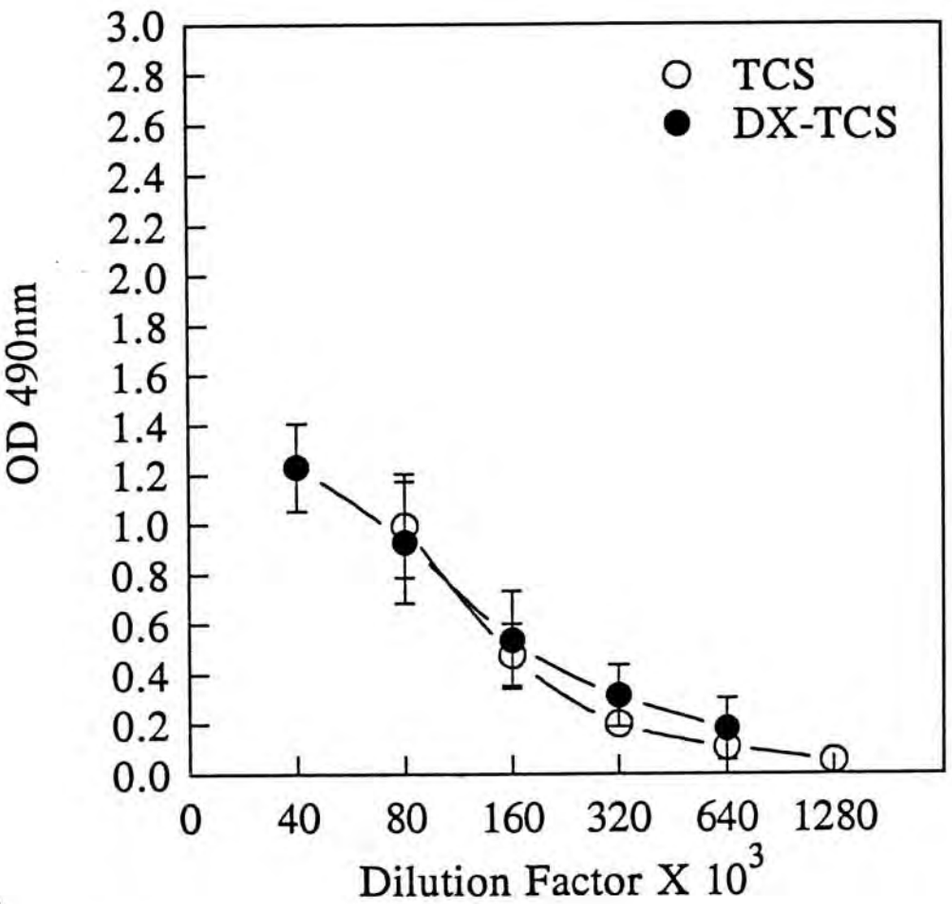


Fig. 8.11 Two-fold serial dilution curves of different mouse sera on TCS coated microtitre plate for IgG determination (without adjuvant). Values are expressed as means ± SEM (TCS,N=5; DX-TCS, N=5).

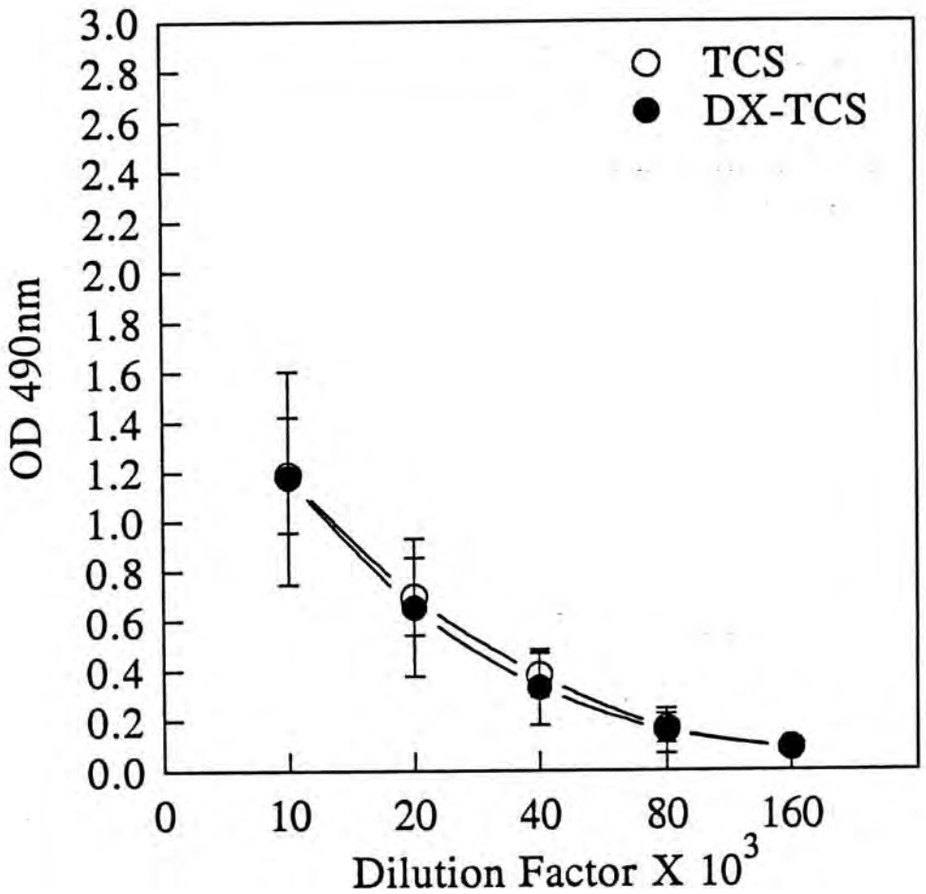


Fig. 8.12 Two-fold serial dilution curves of different mouse sera on DX-TCS coated microtitre plate for IgG determination (without adjuvant). Values are expressed as means ± SEM (TCS,N=5; DX-TCS, N=5).

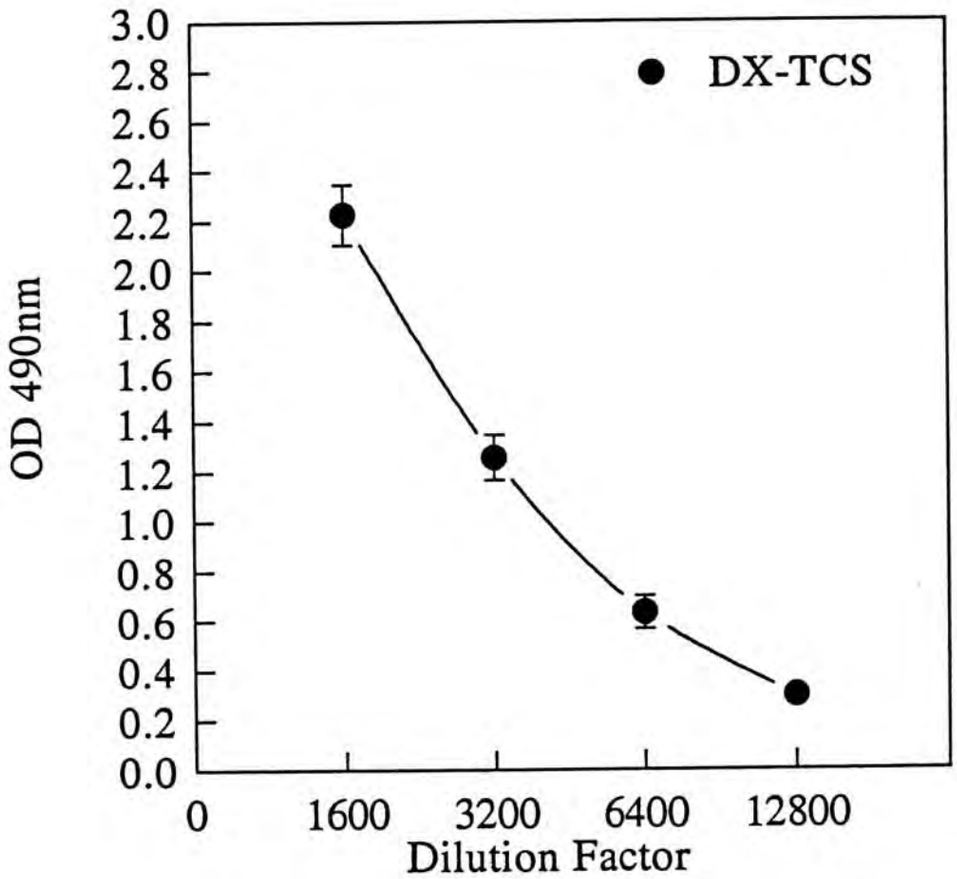


Fig. 8.13 Two-fold serial dilution curves of different mouse sera on dextran coated microtitre plate for IgG determination (without adjuvant). Values are expressed as means ± SEM (DX-TCS, N=5).

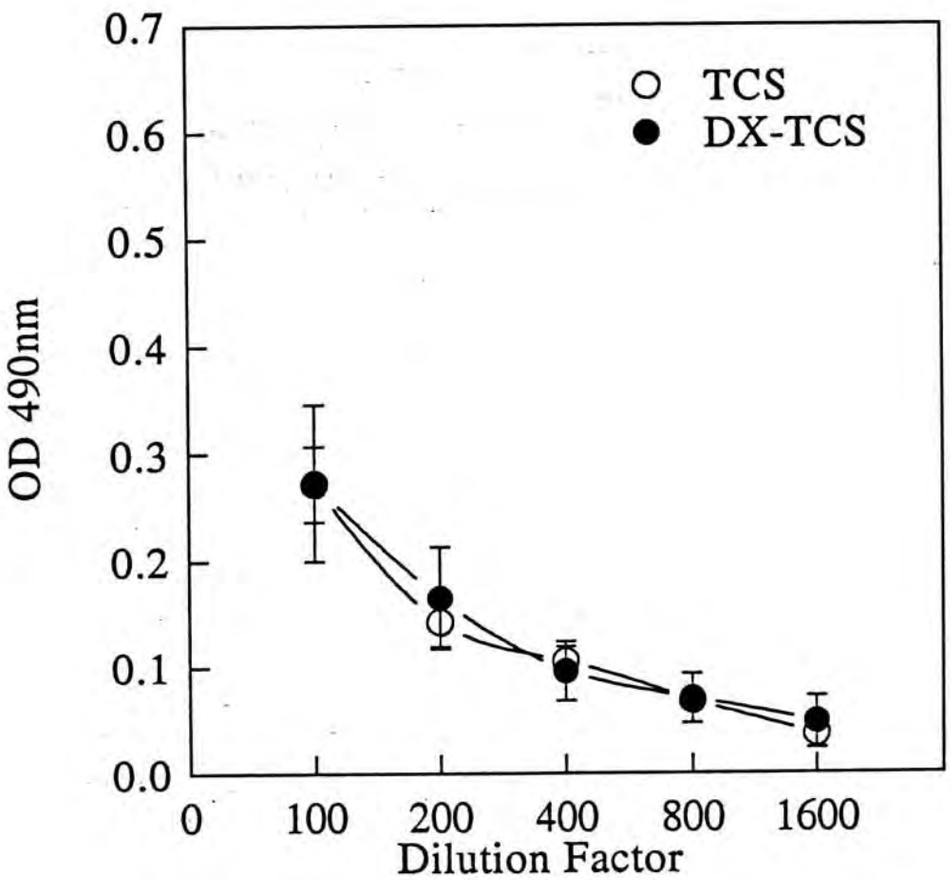


Fig. 8.14 Two-fold serial dilution curves of different mouse sera on TCS coated microtitre plate for IgE determination (without adjuvant). Values are expressed as means ± SEM (TCS,N=5; DX-TCS, N=5).

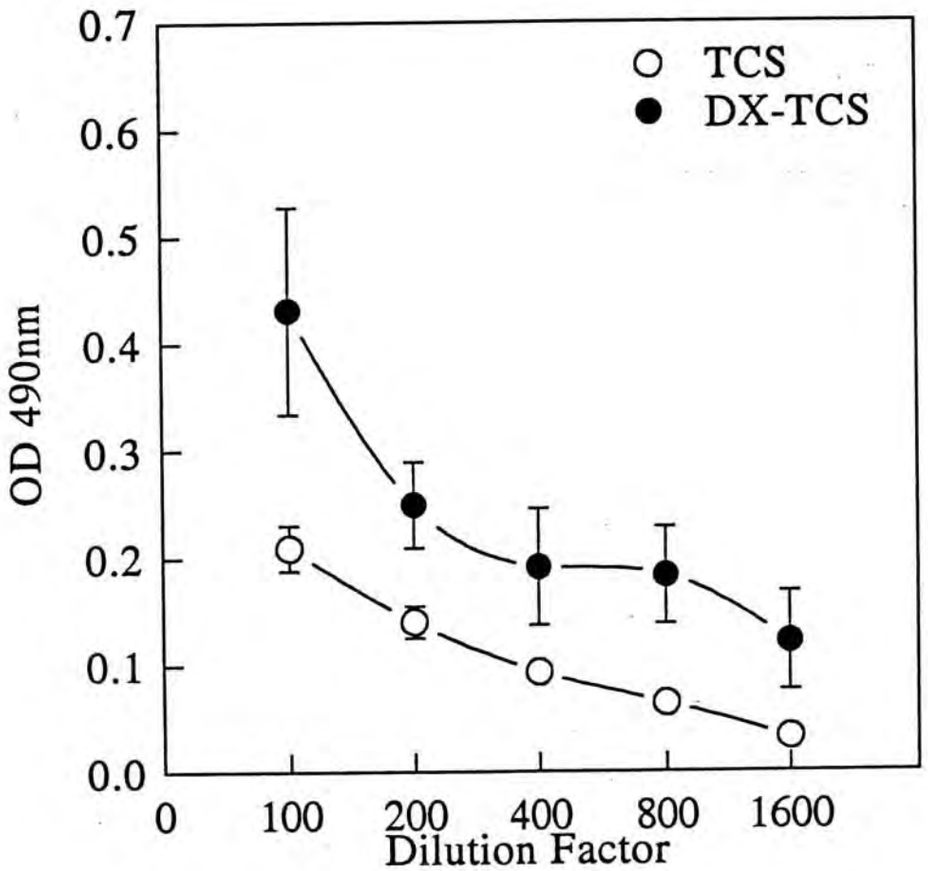


Fig. 8.15 Two-fold serial dilution curves of different mouse sera on DX-TCS coated microtitre plate for IgE determination (without adjuvant). Values are expressed as means ± SEM (TCS,N=5; DX-TCS, N=5).

(a) IgG

	COATING		
	TCS	DX-TCS	Dextran
(1) TCS	+ + + +	+	-
(2) DX-TCS	+ + + +	+	trace
(3) Dextran	_	_	_
(4) Control	_	_	_

(b) IgE

	COATING		
	TCS	DX-TCS	Dextran
(1) TCS	+ +	+	-
(2) DX-TCS	+ +	+ + + +	-
(3) Dextran	_	_	_
(4) Control	_	_	_

Table 8.16 A summary of relative quantity of IgG (upper) and IgE (lower) obtained from Fig. 8.11 - 8.15 in which different mouse sera (without adjuvant) were tested on various antigen coated microtitre plate.

Discussion

Coupling of a molecule to another macromolecule not only prevent its rapid elimination from the circulation, sometimes this approach also has the advantage of reducing the antigenic activity of the parent compound (61,62,63,71). In the competitive radioimmunoassay, the binding curve of DX-TCS to TCS antibodies shifted in a parallel manner to the right of the TCS binding curve. This suggests specific binding of TCS antibodies to DX-TCS but with decreased affinity relative to TCS. This is likely due to the steric hindrance of dextran on the TCS molecule so that some of the antigenic determinants on TCS are not accessible by the antibodies. However, the antibodies can still recognize the complex because some of the epitopes were not masked by dextran or some of the antibodies can possibly overcome the steric hindrance by displacing the flexible side chain away and binds to the complex. This point is further supported by the observation that an increase in DX-TCS binding affinity towards that of TCS after the dextran moiety was digested by dextranase. Free dextran and/or dextranase do not seem to affect the radioimmunoassay. Nevertheless, this binding activities could only be partially reversed. This may be due to the incomplete digestion of dextran by dextranase or the inability of the enzyme to completely digest the periodate oxidized dextran which was different from native dextran.

Apart from the study of antibodies binding characteristic of DX-TCS, its immunogenicity was also tested and compared with the native compound. Numerous examples have shown that after coupling the protein molecule to dextran, the antigen reactivity was lowered (61,62,63,71). In the present study, both TCS and DX-TCS were not different from each other in inducing TCS specific IgG and IgE when no adjuvant was used. However, when the immune system was stimulated by using adjuvant which is a non-specific immunostimulator, substantial difference was seen in the immunogenicity between TCS and DX-TCS in inducing TCS specific antibodies. This phenomena was more prominent in IgE production in which a eight fold difference was observed. This suggests coupling sterically protect TCS from immune recognition. During the process, the antigen is first taken up by the antigen presenting cells. The partially degraded antigen is then presented on the cell surface in associated with MHC molecules. This is followed by the T cell recognition in which its help is probably necessary for a strong immune response. If the dextran moiety is small, T and B cell collaboration is allowed to take place and would result in strong immune response. However, a large dextran moiety probably interferes with the recognition and hence result in a weaker response. Indeed, Seppälä *el al* showed that the decreased immunogenicity was related to the size of dextran molecule used. When small size dextran molecule (e.g. 1 to 4 kDa) was used to couple the protein, a stronger immunogenic response was elicited. However, when the size of dextran was increased to 40 kDa, the antigenicity was greatly reduced (62).

Nevertheless, the most important purpose of this study is to see whether coupling can actually reduce the immunogenicity of the compound. This can be concluded from the comparison between TCS sera on TCS microtitre plate and DX-TCS sera on DX-TCS microtitre plate. When adjuvant was used, DX-TCS was found to elicit much less IgG and IgE that could bind to the conjugate than that of TCS. Therefore, coupling seems to has the beneficial effect in reducing the immunogenicity of the compound in terms of the production of IgG and IgE. Also, one interesting finding is that the IgG elicited by DX-TCS in fact binds more readily to TCS plate than to DX-TCS plate. This suggests that some of the TCS specific IgG generated by DX-TCS immunization could not react with the conjugate probably due to the masking of the epitope(s) by dextran in the intact conformation of DX-TCS. This is analogous to the 'canyon hypothesis' applied to the construction of HIV vaccine. In this hypothesis, it suggests that the binding site of the epitope may be inaccessible to the antibodies generated in the immune response due to steric hinderance. It is because the binding site recognized by the antibodies may be physically located in a deep cleft, or groove, surrounded by steep walls that made the antigen-antibody interaction difficult (105).

Trace amount of the antibodies generated after DX-TCS injection (with adjuvant) was directed towards the dextran component as shown by a positive result on the dextran coated microtitre plate. Normally dextran is non-immunogenic in this

strain of mice since injection of dextran alone failed to produce anti-dextran antibodies. However, the production of anti-dextran antibodies by the protein conjugate of dextran was due to the haptenic effect with the help of T lymphocytes.

In summary, the conjugate was shown to possess lower antigen reactivity as demonstrated by generating less DX-TCS specific IgG and IgE when compared to the parent compound. Therefore, DX-TCS is more superior to TCS in this aspect and may reduce the risk of hypersensitivity reactions when giving to the patients.

Chapter 9: General Discussion

Trichosanthin (TCS) is a purified plant protein isolated from the root tubers of *Trichosanthes kirilowii* (1,2,3). It possess wide range of biological and pharmacological activities. In China, TCS has been used in the treatment of ectopic pregnancy, hydatidiform mole, choriocarcinoma and mid-term abortion (5,6,7). The mechanism is believed to be mediated through its ribosome inactivating property (18,19,20). Recently, it was shown that TCS could inhibit the replication of human immunodeficiency virus (HIV-1) *in vitro* (47,48,49). Despite all these therapeutic applications of the protein, its usage is hampered by two major limitations. First, TCS is a small molecular weight protein (26 kDa) which can be easily filtered through the kidney and lost in urine. This means that patients have to receive frequent repeated doses in order to attain a certain plasma concentration of the drug. This is inconvenient to the patients and repeated doses would also increase the risk of hypersensitivity response. Second, TCS itself is antigenic and cause anaphylaxis in some patients (93,94). In view of these shortcomings, attempt has been made to increase the circulatory survival of TCS and to minimize its allergic side effect simultaneously. The approach is to couple the TCS molecule to a soluble biocompatible polysaccharide dextran T40. The molecular size will be larger and the complex can escape glomerular filtration. Steric hinderance might mask some epitopic site and reduce antigenicity of the molecule.

This study can be divided into three major parts; (1) pharmacokinetic study of TCS and its conjugate; (2) biological evaluation of DX-TCS and (3) immunological study of DX-TCS. To start this study, a sensitive detection method has to be developed to quantify TCS specifically. For this purpose, a radioimmunoassay was successfully developed to detect TCS in plasma and urine in nanogram range. In the pharmacokinetic study, kidney was found to be the major organ of TCS elimination leading to very short plasma half-life of the protein. Coupling of TCS to dextran was achieved by the periodate oxidation method (or dialdehyde method). After coupling, the conjugate has a much reduced renal and non-renal clearance rate due to the expanded molecular size. As a consequent, lesser dosage of the drug which is toxic in nature may be used. It may also avoid the requirement of repeated doses and hence reduces the risk of hypersensitivity reaction. It was also found that TCS was reabsorbed by renal tubular cells. The reabsorption process is saturable and is likely to be the same as the common endocytotic process for other low molecular weight proteins. Excretion of TCS could be increased by simultaneous infusion of another filterable proteins such as lysozyme or haemoglobin.

After injection of TCS, there was a significant decrease in glomerular filtration rate (GFR) suggesting renal toxicity. However, it is possible that GFR did not change significantly after injection of DX-TCS. The entry of TCS into renal tubular cell cause damage. If it can be prevented form reabsorption, renal toxicity can

be avoided. Therefore, coupling of TCS to dextran not only prolonged the plasma half-life, it also served to prevent renal toxicity.

The DX-TCS conjugate would not be useful if it lost its activity. Therefore it is essential to evaluate the biological activity of the conjugate. When compared to the native compound, the biological activities were well preserved as manifested by the mid-term abortifacient activity, anti-tumour activity and its immunosuppressive ability. However, the potency of the complex was decreased. This may be due to the blocking of active site(s) by dextran molecule or the conformation of the active site(s) has been altered after modification. In spite of this, the toxicity of TCS was also decreased as seen in a reduced mortality rate in the treated pregnant mice and this further improved the therapeutic usage of the drug. Evidence suggest that after coupling the cellular entry of DX-TCS into non-phagocytotic cells was impaired. Reduction in toxicity may due to the cellular penetration of the conjugate was limited to those of the phagocytotic target cells but not other normal cells.

As described earlier, one of the limitations of TCS is its antigenic side effect. Coupling may have the advantage of sterically protect the molecule from immune recognition. In fact, this was demonstrated by the following two findings. Firstly, in the antibody binding study of DX-TCS, the binding affinity of the conjugate towards TCS antibodies was decreased. Secondly, in the immunogenicity study, DX-TCS was

shown to elicit less IgG and IgE that react with the complex when compared with the native molecule. Therefore, it could be concluded that the conjugate has reduced antigenicity when compared to the native compound. This further improves the therapeutic usage of the drug by reducing the risk of developing hypersensitivity.

Finally, for the future follow-up research, several extended studies may be performed. The toxicity of TCS was decreased after coupling to dextran. This may be related to the difference in cellular penetration ability of DX-TCS to phagocytotic and non-phagocytotic cells. Therefore, in order to substantiate our hypothesis that the conjugate, like its parent compound is still able to enter the target cells to achieve its action, it is essential to delineate the detail mechanism of cellular entry of TCS and its conjugate. Up to now, very little is known about the phenomena of cellular penetration and kinetics of the single chain ribosome inactivating protein. Therefore, by applying the fluorescence imaging technique and electron-microscopic immunocytochemistry, the process of penetration between native and modified compound on phagocytotic and non-phagocytotic cells can be compared. Moreover, another setback of this coupling method is that we do not know the exact position that the dextran binds to the TCS molecule. This will lead to the production of a population of conjugates with the dextran binding to different active or immunogenic site(s) on the molecule. By using another method of coupling known as the alkylation method, the dextran can bind specifically to the sulfhydryl residue on the protein

molecule. Although the native TCS molecule lacks the sulfhydryl group for coupling, it may prove possibe to induce a site mutation on the TCS gene which has been recently cloned so that the new recombinant TCS possess the amino acid cysteine (and hence sulfhydryl group) on the surface of the molecule. Therefore by combining the present approach with the advent of molecular biology, we can specifically attach the dextran to different position and this helps to elucidate the exact location of active and immunogenic site(s) of the molecule.

References

1. Yeung HW, Wong DM, Ng TB, Li WW. Purification of three isolectins from root tubers of *Trichosanthes kirilowii* (Tian hua fen). Int J Pept Protein Res 1986;**27**:325-323.

2. Wang Y, Qian RQ, Gu ZW, Jin SW, Zhang LQ, Xia ZX, Tian GY, Ni CZ. Scientific evaluation of Tian Hua Fen: history, chemistry and application. Pure Appl Chem 1986;**58**:789-798.

3. Wang YH, Liu JF, Zhu LX. Preliminary studies on purification and characterization of an abortifacient plant protein, trichosanthin. Acta Zool Sinica 1976;**22**:137-143.

4. Wang SF, Jin SW, Tien KY. The investigation and application of Tian hua-fen proteins. Scientific Publications, Beijing, China, 1979.

5. Liu GW, Liu FY, Li YJ *et al.* A summary of 402 cases of termination of early pregnancy with crystalline preparations of trichosanthin. In: Advances in Chinese medicinal materials research. Eds., Chang HM, Yeung HW, Tso WW, Koo A. Singapore: World Scientific Publ. Co., 1985, pp.327-323.

6. Jin YC. Clinical study of trichosanthin. In: Advances in Chinese medicinal materials research. Eds., Chang HM, Yeung HW, Tso WW, Koo A. Singapore: World Scientific Publ. Co., 1985, pp.319-326.

7. Huang Y. A clinical study on treatment of malignant trophoblastic neoplasia with trichosanthin. Chung Hsi I Chieh Ho Tsa Chih 1987;**7**:154-155.

8. Yeung HW, Poon SP, Li WW. Isolation of an immunosuppressive protein from the Chinese herb, Tian-hua-fen (*Trichosanthes kirilowii*). Int J Immunopharmacol 1980;**2**:220.

9. Wang Y. Chemistry of trichosanthin, a new biologically active plant protein. In: Advances in Chinese medicinal materials research. Eds., Chang HM, Yeung HW, Tso WW, Koo A. Singapore: World Scientific Publ. Co., 1985, pp.289-295.

10. Pan KZ, Zhang YM, Lin YJ *et al.* The secondary structure of trichosanthin. In: Advances in Chinese medicinal materials research. Eds., Chang HM, Yeung HW, Tso WW, Koo A. Singapore: World Scientific Publ. Co., 1985, pp.297-303.

11. Collins EJ, Robertus JD, LoPresti ML, Stone KL, Williams KR, Wu P, Hwang K, Piatak M. Primary amino acid sequence of α-Trichosanthin and molecular models for Abrin A-chain and α-Trichosanthin. J Biol Chem 1990;**265:**8865-8669.

12. Kubota S, Yeung HW, Yang JT. Conformation of abortifacient proteins: trichosanthin, α-momorcharin and ß-momorcharin. Biochim Biophys Acta 1986;**871:**101-106.

13. Pan KZ, Zhang YM, Lin YJ, Wu CW, Zheung A, Chen YZ, Dong C, Ma XQ, Wang YP, Wu S, Zhang MG, Chen SZ, Xia ZX, Tian GY, Ni CZ, Fan ZC, Ma YL, Sun XX. The backbone structure of Trichosanthin. In: Advances in Chinese medicinal materials research. Eds., Chang HM, Yeung HW, Tso WW, Koo A. Singapore: World Scientific Publ. Co., 1985, pp.305-309.

14. Chow TP, Feldman RA, Lovett M, Piatak M. Isolation and DNA sequence of a gene encoding α-Trichosanthin, a type I ribosome-inactivating protein. J Biol Chem 1990;**265:**8665-8669.

15. Shaw PC, Yung MH, Zhu RH, Ho WK, Ng TB, Yeung HW. Cloning of trichosanthin cDNA and its expression in *Escherichia coli*. Gene 1991;**97:**267-272.

16. Olsnes S, Pihl A. In: Molecular Actions of Toxins and Viruses. Eds., Cohen P, Van HS. Elsevier Science Publ. Co. Inc., New York, 1982, pp.51-105.

17. Stripe F, Barbieri L. Ribosome-inactivating proteins up to date. FEBS Lett 1986;**195:**1-8.

18. Yeung HW, Li WW, Feng Z, Barbieri L, Stripe F. Trichosanthin, α-momorcharin and ß-momorcharin: identity of abortifacient and ribosome-inactivating proteins. Int J Pept Protein Res 1988;**31:**265-268.

19. Wang Q, Zhang Z, Wang J, Xie H, Yang Z. Trichosanthin has potent inhibiting activity of protein synthesis in a cell-free system and its antibody-conjugate exhibits potent cytotoxicity to tumor cells *in vitro*. Shih Yen Sheng Wu Hsueh Pao 1987;**20:**515-519.

20. Maraganore JM, Joseph M, Bailey MC. Purification and characterization of trichosanthin: Homology to the ricin A chain and implications as to mechanism of abortifacient activity. J Biol Chem 1987;**262:**11628-11633.

21. Zhang X, Wang J. Homology of trichosanthin and ricin A-chain. Nature (Lond.) 1986;**321:**477-478.

22. Kubota S, Yeung HW, Yang JT. Conformation similarities of ricin A-chain and trichosanthin. Int J Pept Protein Res 1987;**30:**646-665.

23. Pan KZ, Lin YJ, Fu Z *et al.* Three-dimensional structure of trichosanthin molecule. Sci China [B] 1987;**30:**386-395.

24. Montfort W, Villafranca JE, Monzingo AF, Ernst SR, Katzin B, Reutenber E, Xuong NH, Hamlin R, Robertus JD. The three-dimensional structure of ricin at 2.8A. J Biol Chem 1987;**262:**5398-5403.

25. Endo Y, Mitsui K, Motizuki M, Tsurugi K. The mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. J Biol Chem 1988;**262:**5908-5912.

26. Stirpe F, Bailey S, Miller SP, Bodley JW. Modification of ribosomal RNA by ribosome-inactivating proteins from plants. Nucleic Acids Res 1988;**16:**1349-1367.

27. Brigotti M, Rambelli F, Zamboni M, Montanaro L, Sperti S. Effect of α-sarcin and ribosome-inactivating proteins on the interaction of elongation factors with ribosomes. Biochem J 1989;**257:**723-727.

28. Endo Y, Tsurugi K, Yutsudo T, Takeda Y, Ogasawara T, Igarashi K. Site of action of a vero toxin (VT2) from *Escherichia coli* O157:H7 and of shiga toxin on eukaryotic ribosomes. Eur J Biochem 1988;**171:**45-50.

29. Cheng KF. Midtrimester abortion induced by Radix trichosanthis: morphologic observations in placenta and fetus. Obstet Gynecol 1982;**59:**494-498.

30. Lau IF, Saksena SK, Chang MC. Further studies on the trichosanthin induced termination of pregnancy. Contraception 1980;**21:**77-86.

31. Chang MC, Saksena SK, Lau IF, Wang YH. Induction of mid-term abortion by trichosanthin in laboratory animals. Contraception 1979;**19:**175-184.

32. Xiong YZ, Pan P, Xu SY, Zhang J, Wang YH, Zuo JK, Gu Z. Study on the effects of Trichosanthin on human trophoblast cells *in vitro*. Acta Zool Sinica 1976;**22:**172-179.

33. Hsu KJ, Jiang WS, Chen YF, Huang MB. Studies on the site of action of induced abortion by Trichosanthin. Acta Zool Sinica 1976;**22:**149-155.

34. Wang YT, Ju RM, Huang JH, Hsu KC. Investigations on the injurious effects of the abortifacient Trichosanthin to monkey placental villi. Acta Zool Sinica 1976;**22:**156-165.

35. Anonymous. Studies on the mechanisms of abortion induced by trichosanthin. Sci China [B] 1976;**19:**811-827.

36. Law LK, Tam PPL, Yeung HW. Effects of α-trichosanthin and α-momorcharin on the development of peri-implantation embryos. J Reprod Fertil 1983;**69:**597-604.

37. Yao JF. The analysis of 106 cases of malignant trophoblastic tumours treated by combination of Chinese and Western medicine. Jiangsu Med 1978;**6:**5-7.

38. Tsao SW, Yan YT, Yeung HW. Selective killing of choriocarcinoma cells in vitro by trichosanthin, a plant protein purified form root tubers of the Chinese medicinal herb, *Trichosanthes kirilowii*. Toxicon 1986;**24:**831-840.

39. Wong YF, Wong WSF, Chew EC, Chang MZA. A morphological study of effects of trichosanthin on cultured choriocarcinoma cells. Med Sci Res 1990;**18:**379-381.

40. Wong YF, Wong WSF, Yeung HW, Chang MZA. Effects of the combination of trichosanthin with Adriamycin and cisplatin on cultured tumour cells. Med Sci Res 1989;**17:**167-169.

41. Wong YF, Wong WSF, Chen XG, Zeng XM, Chang MZ. Evaluation of trichosanthin against Xenografts of human choriocarcinoma. Med Sci Res 1990;**18:**95-96.

42. Wong YF, Wong WSF, Chang MZA. Effects of trichosanthin on cell growth, cell cycle phase distribution and macromolecule synthesis in human choriocarcinoma cells. Med Sci Res 1990;**18:**383-384.

43. Tsao SW, Ng TB, Yeung HW. Toxicities of trichosanthin and α-momorcharin, abortifacient proteins form Chinese medicinal plants, on cultured tumor cell lines. Toxicon 1990;**28:**1183-1192.

44. Vitetta ES, Fulton RJ, May RD, Till M, Vhr JW. Redesigning nature's position to create anti-tumor reagents. Science (Washington DC) 1987;**238:**1098-1104.

45. Pastan I, Willingham MC, FitzGerald DJP. Immunotoxins. Cell 1986;**47:**641-648.

46. Wang QC, Ying WB, Xie H, Zhang ZC, Yang ZH, Ling LQ. Trichosanthin-monoclonal antibody conjugate specifically cytotoxic to human hepatoma cells *in vitro*. Cancer Res 1991;**51:**3353-3355.

47. McGrath MS, Hwang KM, Caldwell SE, Gaston I, Luk KC, Wu P, Ng VL, Crowe S, Daniels J, Marsh J, Deinhart T, Lekas PV, Vennari JC, Yeung HW, Lifson JD. GLQ 223: an inhibitor of human immunodeficiency virus replication in acutely and chronically infected cells of lymphocyte and mononuclear phagocyte lineage. Proc Natl Acad Sci USA 1989;**86:**2844-2848.

48. Ferrari P, Trabaud MA, Rommain M, Mandine E, Zalisz R, Desgranges C, Smets P Laboratories Cassenne, Centre de Recherches, Immunologiques Roussel-Vclaf, Cergy Pontoise, France. Toxicity and activity of purified trichosanthin. AIDS (US) 1991;**5:**865-870.

49. McGrath MS, Santulli S, Gaston I. Effects of GLQ 223 on HIV replication in human monocyte/macrophages chronically infected *in vitro* with HIV. AIDS Res Hum Retroviruses 1990;**6:**1039-1043.

50. Kahn JO, Kaplan LD, Gambertoglio JG, Bredesen D, Arri CJ, Turin L, Kiborti T, Williams RL, Lifson JD, Volberding PA. The safety and pharmacokinetics of GLQ 223 in subjects with AIDS and AIDS-related complex: a phase I study. AIDS (US) 1990;**4:**1197-1204.

51. Byers VS, Levin AS, Waites LA, Starrett BA, Mayer RA, Clegg JA, Price MR, Robins RA, Delaney M, Baldwin RW. A phase I/II study of trichosanthin treatment of HIV disease. AIDS (US) 1990;**4:**1189-1196.

52. Li MX, Yeung HW, Pan LP, Chan SI. Trichosanthin, a potent HIV-1 inhibitor, can cleave supercoiled DNA *in vitro*. Nucleic Acids Res 1992;**19:**6309-6312.

53. Leung KN, Yeung HW, Leung SO. The immunomodulatory and antitumor activities of trichosanthin - an abortifacient protein isolated from Tian-hua-fen (*Trichosanthes kirilowii*). Asian Pac J Allergy Immunol 1986;**4:**111-120.

54. Yeung HW, Poon SP, Ng TB, Li WW. Isolation and characterization of an immunosuppressive protein form *Trichosanthes kirilowii* root tubers. Immunopharmacol Immunotoxicol 1987;**9:**25-46.

55. Zheng SS, Li WW, Yeung HW, Wu AR. Kinetics of IgE antibody response to Trichosanthin, α-momorcharin and ß-momorcharin in mice. Chin Med J 1991;**104:**292-299.

56. Gu H, Yeh M, Yao Z. Investigation of antigenic determinants of Trichosanthin by antibody competitive binding assay. Shih Yen Sheng Wu Hsueh Pao 1986;**19:**121-129.

57. Sherwood RF, Baird JK, Atkinson T, Wiblin CN, Rutter DA, Ellwood DC. Enhanced plasma persistence of therapeutic enzymes by coupling to soluble dextran. Biochem J 1977;**164:**461-464.

58. Marshall JJ, Humphreys JD, Abramson SL. Attachment of carbohydrate to enzymes increases their circulatory lifetimes. FEBS lett 1977;**83:**249-252.

59. Rutter DA, Wade HE. The influence of the iso-electric point of L-asparaginase upon its persistence in the blood. Br J Exp Pathol 1971;**52:**610-614.

60. Broome JD. Antilymphoma activity of L-asparaginase *in vivo*: clearance rates of enzyme preparations from guinea pig serum and yeast in relation to their effect on tumor growth. J Natl Cancer Inst 1965;**35:**967-974.

61. King TP, Kochoumian L, Ishizaka K, Lichtenstein LM, Norman PS. Immunochemical studies of dextran coupled ragweed pollen allergen, antigen E. Arch Biochem Biophys 1975;**169:**464-473.

62. Seppälä I, Makela O. Antigenicity of dextran-protein conjugates in mice: effect of molecular weight of the carbohydrate and comparison of two modes of coupling. J Immunol 1989;**143:**1259-1264.

63. Fagnani R, Hagan MS, Bartholomew R. Reduction of immunogenicity by covalent modification of murine and rabbit immunoglobulins with oxidized dextrans of low molecular weight. Cancer Res 1990;**30:**3638-3643.

64. Aspinall, GO. Polysaccharides. Pergamon Press, New York, 1970.

65. Kabat, EA. The upper limit for the size of the human anti-dextran combining site. J Immunol 1960;**84:**82-85.

66. Tam SC, Blumenstein J, Wong JTF. Soluble dextran-hemoglobin complex as a potential blood substitute. Proc Natl Acad Sci USA 1976;**73:**2128-2131.

67. Blumenstein J, Tam SC, Chang JE, Wong JTF. Experimental transfusion of dextran-hemoglobin. In: Blood Substitutes and plasma expanders. Eds., Jamieson GA, Greenwalt TJ. Alan R. Liss Inc., New York, 1978, pp.205-212.

68. Tam SC, Blumenstein J, Wong JTF. Blood replacement in dogs by dextran-hemoglobin. Can J Biochem 1978;**56:**981-984.

69. Cunnington PG, Jenkins SN, Tam SC, Wong JTF. Oxygen binding and immunological properties of complexes between dextran and animal hemoglobin. Biochem J 1981;**193:**261-266.

70. Wade HE, Rutter DA. Asparaginase-treatment for leukaemia. Sci J 1970;**6:**62-70.

71. Wileman TE, Foster RL, Elliott PNC. Soluble asparaginase-dextran conjugates show increased circulatory persistence and lowered antigen reactivity. J Pharm Pharmacol 1986;**38:**264-271.

72. Wileman TE, Bennett M, Lilleymann J. Potential use of an asparaginase-dextran conjugate in acute lymphoblastic leukaemia. J Pharm Pharmacol 1983;**35:**762-765.

73. Melton RG, Wiblin CN, Foster RL, Sherwood RF. Covalent linkage of carboxyl peptidase G_2 to soluble dextran - I. Properties of conjugates and effects on plasma persistence in mice. Biochem Pharmacol 1987;**36:**105-112.

74. Melton RG, Wiblin CN, Baskerville A, Foster RL, Sherwood RF. Covalent linkage of carboxyl peptidase G_2 to soluble dextran - II. *In vivo* distribution and fate of conjugates. Biochem Pharmacol 1987;**36:**113-121.

75. Rosemeyer H, Körnig E, Seela F. Adenosine deaminase covalently linked to soluble dextran. The effect of immobilization on thermodynamic and kinetic parameters. Eur J Biochem 1982;**122:**375-380.

76. Ko WH, Wong CC, Yeung HW, Yung MH, Shaw PC, Tam SC. Increasing the plasma half-life of trichosanthin by coupling to dextran. Biochem Pharmacol 1991;**42:**1721-1728.

77. Sambrook J, Fritsch EF, Maniatis T. Screening expression libaries with antibodies and oligonucleotides. In: Molecular cloning, A laboratory mannual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, chap. 12.

78. Hunter WM, Greenwood FC. Preparation of Iodine-131 labelled human growth hormone of high specific activity. Nature (Lond.) 1962;**194:**495-496.

79. Mogensen CE, Sϕlling K. Studies on renal tubular protein reabsorption: partial and near complete inhibition by certain amino acids. Scand J Clin Lab Invest 1977;**37:**477-486.80.

80. Philip HJ. Dye exclusion tests for cell viability. In: Tissue culture. Eds., Kruse PF, Patterson MK. Academic Press, New York, 1973, pp.406-408.

81. Flemming C, Gabert A, Roth P. Synthese und eigenschaften trägerfixierter enzyme - I. Kovalente binding von trypsin an dialdehydzellulose. Acta Biol Med Germ 1973;**30:**177-182.

82. Sanderson CJ, Wilson DV. A simple method for coupling proteins to insoluble polysaaccharides. Immunology 1971;**20:**1061-1065.

83. Gibaldi M, Perrier D. Pharmacokinetics. In: Drugs and the Pharmaceutical Science, Vol. 15, 2nd ed., Marcel Dekker, New York, 1982.

84. Bernard A, Amor AO, Viau C, Lauwerys R. The renal uptake of proteins: a non-selective process in conscious rats. Kidney Int 1988;**34:**175-185.

85. Cojocel C, Franzen-Sieveking M, Beckmann G, Baumann K. Inhibition of renal accumulation of lysozyme (basic low molecular weight protein) by basic protein and other basic substances. Pflügers Arch 1981;**390:**211-215.

86. Foulkes EC. Apparent competition between myoglobin and metallothionein for renal reabsorption. Proc Soc Exp Biol Med 1978;**195:**321-323.

87. Foulkes EC. Renal tubular transport of cadmium-metallothionein. Toxicol Appl Pharmacol 1978;**45:**505-512.

88. Cheng TS, Ko WH, Swaminathan R, Tam SC. Effect of lysine on hemolysis-induce kidney damage. J Lab Clin Med 1992 (in press).

89. Drabkin DL, Austin JH. Spectrophotometric studies. II. Preparations from washed blood cells; nitric oxide hemoglobin and sulfhemoglobin. J Biol Chem 1935;**112:**51-65.

90. Waldmann TA, Strober W, Mogielnicki RP. The renal handling of low molecular weight protein. J Clin Invest 1972;**51:**2162-2174.

91. Bunn HF, Jandl JH. The renal handling of hemoglobin. II. Catabolism. J Exp Med 1969;**129:**925-934.

92. Malis CD, Racusen LC, Solez K. Nephrotoxicity of lysine and of a single dose of aminoglycoside in rats given lysine. J Lab Clin Med 1984;**103:**660-676.

93. Anonymous. A clinical and ultrastructural study of combination treatment of malignant mole using trichosanthin. Commun Med 1974;**420:**45-48.

94. Zhuang IQ, Sun SC. Serum antibody to Radix trichosanthis after its use for terminating midterm pregnancy. Chinese J Obstet Gynecol 1974;**14:**122-125.

95. Poznansky MJ, Shandling M, Salkie MA, Elliott J, Lan E. Advantages in the use of L-asparaginase-albumin polymer as an antitumor agent. Cancer Res 1982;**42:**1020-1025.

96. Bendich A, Kafkewitz A, Abuchowski A, Davis FF. Immunological effects of native and polyethylene glycol-modified asparaginase from *Vibrio succinogenes* and *Escherichia coli* in normal and tumour-bearing mice. Clin Exp Immunol 1982;**48:**273-278.

97. Girard J, Greenwood FC. The absence of intact growth hormone in urine as judged by radioimmunoassay. J Endocr 1968;**40:**493-503.

98. Srivastava MC, Tompkins CV, Sonken PH, Nabarro J. Antisera for radioimmunoassay. In: Radioimmunoassay methods. Eds., Kirkham KE, Hunter WM. Churchill livingstone, Edinburgh and London, 1971, pp.211-215.

99. Tamhane AC. Multiple comparisons in model I one-way ANOVA with unequal variances. Commun Statistics 1977;**A6:**15-32.

100. Chan Y, Tong MK, Lau MJ. 238 cases of malignant hydatid mole treated by the combination of Chinese and Western medicine. Shanghai J Chin Med 1982;**3:**30-35.

101. Schnermann J, Briggs J. Role of the renin-angiotensin system in tubuloglomerular feedback. Fed Proc 1986;**45:**1426-1430.

102. Persson BE, Marsh DJ. GFR regulation and flow-dependent electrophysiology of early distal tubule in Amphiuma. Am J Physiol 1987;**253:**F263-268.

103. Blantz RC, Pelayo JC. A functional role for the tubuloglomerular feedback mechanism. Kidney Int 1984;**25:**739-746.

104. Ichikawa I. Direct analysis of the effector mechanism of the tubuloglomerular feedback system. Am J Physiol 1982;**243:**F447-455.

105. Rossmann MG. Antiviral agents targeted to interact with viral capsid proteins and a possible applications to the human immunodeficiency virus. Proc Natl Acad Sci 1988;**13:**4625-4627.

106. Harris JM, Kalmus H, West GB. Genetic control of the anaphylactoid reaction in rats. Genet Res 1963;**4:**346-355.

107. Truscello A, Gaggeler HP, Rossier BC. Thyroid hormone antagonizes an aldosterone-induced protein: A candidate mediator for the late mineralocorticoid response. J Membr Biol 1986;**89:**173-183.